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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/11, 15/12, C07K 14/47, C12N 9/64, C12Q 1/68, A61K 48/00

A2

(11) International Publication Number: **WO 98/39429**

(43) International Publication Date: 11 September 1998 (11.09.98)

(21) International Application Number: PCT/IL98/00102

(22) International Filing Date: 3 March 1998 (03.03.98)

(30) Priority Data:
08/810,712 3 March 1997 (03.03.97) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: COMPOSITIONS FOR TREATMENT OF DISORDERS INVOLVING PROGRAMMED CELL DEATH

(57) Abstract

Use of a DNA sequence capable of inducing programmed cell death, in the preparation of a pharmaceutical composition for use in the treatment of a disease or a disorder associated with metastasizing pathological cell growth. Also described is the use of a DNA sequence capable of promoting non–cytokine–induced programmed cell death, in the preparation of a pharmaceutical composition useful in the treatment of a disease or a disorder associated with uncrontrolled pathological cell growth, or in the treatment of a disease or a disorder associated with non–cytokine induced programmed cell death.

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COMPOSITIONS FOR TREATMENT OF DISORDERS INVOLVING PROGRAMMED CELL DEATH

FIELD OF THE INVENTION

The present invention relates to the field of programmed cell death genes and their products.

BACKGROUND OF THE INVENTION

One of the factors which determines the proliferation state of cells is the balance between the growth-promoting effects of proto-oncogenes, and the growth-constraining effects of tumor-suppressor genes.

One mechanism by which these tumor-suppressor genes exert their growth-constraining effect is by inducing the cell to undergo a physiological type of death. Such a controlled cell death is evident in a multitude of physiological conditions including metamorphosis, synaptogenesis of neurons, death of lymphocytes during receptor repertoire selection, controlled homeostasis in the bone-marrow and other proliferative tissues, and others. Such cell death is regulated by the interaction of the cell with other cells or with cell products, for example through the activity of suitable cytokines.

Genetic mutations that inactivate the suppressor genes, liberate the cell from normal growth constraints imposed by other cells or by cytokines, resulting in an uncontrolled growth or viability of the cell without any relation to external signals. This uncontrolled growth is a step in tumorigenesis.

To date, only a few tumor-suppressor genes have been fully characterized including the *retinoblastoma (Rb)* gene, *p53, DCC, NM23 WT-1, NF-1, APC*, and *ras suppressor* genes. A mutation in either of the above genes, probably in both alleles, which leads to either blockage of expression, or production of a faulty protein, hampers the normal control of growth and viability of cells and may thus give rise to cancer.

A number of links have been discovered between programmed cell death and the multi-stage process of tumorigenesis. The first discovery was the finding that the Bcl2 gene, activated by the typical chromosomal translocation in human follicular lymphomas, is a suppressor of cell death (Tsujimoto Y., et al., 1985, Nature **315**:340-343). The second link was the finding that p53, the most commonly mutated tumor suppressor gene in various human tumors, functions as a positive mediator of apoptosis. p53 induces cell death in response to different stresses such as gentoxic damage and hypoxia. Thus, the extension of cell viability followed by the accumulation of genetic damage and by the uncontrolled growth of the tumor, are among the mechanisms through which inactivating mutations of p53 promote tumorigenesis (Lowe, S.W., et al., 1993, Nature **362**:847-849). More recently it has been reported that the adenomatous polyposis coli (APC) tumor suppressor gene, that is frequently lost or inactivated in early stages of colorectal cancers, induced the death of colorectal cells in culture (Morin et al., 1996, Proc. Natl. Acad. Sci. **93**:7950-54), thus providing a third link to apoptotic control. In another study, apoptosis in micrometastases was found to be significantly reduced after induction of angiogenesis as a result of a decrease in levels of circulating angiogenic inhibitors (Holmgren, L., et al.

1995, Nature Medicine **1**:149-153). However, very little has been revealed with respect to earlier stages of metastasis such as detachment from the primary tumor, dissemination and invasion processes.

Growth-inhibiting cytokines have a double effect on the target cell. They can either inhibit the proliferation of the cell, and/or give rise to cell death. To date, blockage or activation of expression of known tumor-suppressor genes was shown to counteract or enhance, respectively, cytokines' inhibition of cells' growth (reviewed by A. Kimchi, 1992, *J. Cell Biochem.*, **50**:1-9) but did not have any effect on the death promoting action of cytokines. For example, the growth inhibitory response to cytokines such as TGF-β, was markedly reduced by the inactivation of the *Rb* gene, or the response to IL-6 was enhanced by introducing activated p53 genes (Pietenpol *et al.*, 1990, *Cell*, **61**:777-785; Levy *et al.*, 1993, *Mol. Cell. Biol.*, **13**:7942-7952).

Thioredoxin, a small hydrogen carrier protein, has previously been implicated in the IFN-γ-mediated growth arrest of HeLa cells (Deiss, L.P. and Kimchi, A. 1991, Science **234**:117-120).

SUMMARY OF THE INVENTION

In the following specification, the term *"programmed cell death"* will be used to denote a physiological type of cell death which results from activation of some cellular mechanisms, i.e. death which is controlled by the cell's machinery. Programmed cell death may, for example, be the result of activation of the cell machinery by an external trigger, e.g. a cytokine, which leads to cell death. The term *"apoptosis"* is also used interchangeably with programmed cell death.

The term *"tumor"* in the following specification denotes an uncontrolled growing mass of abnormal cells. This term includes both

primary tumors, which may be benign or malignant, as well as *secondary tumors*, or *metastases* which have spread to other sites in the body.

The present invention is based on the pioneering finding that inhibition of expression of certain genes counteracts the programmed cell death. Namely, as long as these genes function normally, cell death is induced; once the expression of said genes is inhibited, the cell death is inhibited. It follows therefrom that the normal expression product of these genes is involved in programmed cell death, both cytokine-induced and non-cytokine induced. In HeLa cells, the cytokine IFN-γ induces a biphasic process, which comprises an initial cytostatic phase and a subsequent cytotoxic phase (programmed cell death). The novel genes discovered in accordance with the present invention were found to affect only the later, cytotoxic phase. These genes will be referred to herein as *"DAP* (death-associated protein) *genes"*. DNA molecules comprising a coding sequence encoding the expression products of the DAP genes, or expression products having a similar biological activity, will be referred to herein at times collectively as *"DAP DNA molecules"*. The expression products of the DAP DNA molecules will be referred to herein at times collectively as *"DAP products"*.

The present invention is further based on the pioneering finding that metastasizing cells may have a defective internal apoptosis mechanism. Thus, although during metastasis the tumor cells encounter several novel types of apoptotic stimuli, the cells continue to metastasize.

It has further been discovered that by correcting the deficiency which led to the malfunction of the apoptotic mechanism in the cell, the metastatic character of the cell is suppressed.

According to one aspect of the present invention, to be referred to herein as "*the death-promoting aspect*", the above DAP DNA molecules, expression vectors comprising them, or DAP products are used for promoting death of normal or tumor cells and for suppressing the metastatic activity of

tumor cells. A particular application of the death-promoting aspect is in therapy of diseases or disorders associated with uncontrolled, pathological cell growth, e.g. cancer (primary tumors and metastasis), psoriasis, autoimmune diseases and others. The use of DAP DNA molecules in gene therapy or DAP products if produced extracellularly, in accordance with the death-promoting aspect of the invention, may be in conjunction with cytokines, e.g. IFN-γ, in the treatment of cytokine-induced programmed cell death.

According to another aspect of the invention, to be referred to herein as "*the death-preventing aspect*" agents which prevent the expression of said DAP DNA molecules, or agents which antagonize, inhibit or neutralize the DAP products, are used for protecting cells from programmed cell death. Examples of possible applications of the death preventing aspect of the invention are in prevention of cell death in various degenerative neurological diseases, such as Alzheimer's disease or Parkinson's disease, which are associated with premature death of particular subsets of neurons; prevention of death of T-cells in AIDS patients, which death resembles programmed cell death; prevention of rejection-associated cell death in transplants which is believed to result, at least in part, from programmed cell death; protection of normal cells from the cytotoxic effects of certain anti-cancer therapies; etc.

According to a further aspect of the present invention, referred to herein at times as *"the prognostic aspect"*, DAP DNA molecules are used in order to examine individuals suffering from a disease in order to determine whether the disease is related to the defective activity of DAP genes and which therapeutic modalities might be effective. For example, DAP positive cells may be more susceptible to control by chemotherapeutic drugs that work by inducing apoptosis, so that the choice of treatment modalities may made based on the DAP state of the cells.

In accordance with this aspect, the examination is carried out by comparing the sequence of each of the DAP DNA molecules to each of the

respective DAP genes in the individual, or by following RNA and/or protein expression.

For example, the presence and/or composition of DAP DNA molecules may be assessed by Southern blot analysis and/or PCR. The mRNA may be analyzed on Northern blots and/or by reverse-transcription PCR (RT-PCR), followed by sequence analysis and/or by in-situ hybridizations of tissue sections. Protein expression may be monitored in cell extracts by Western analysis, or by in-situ immuno-staining of tissue sections using antibodies to DAP proteins. The absence of a DAP gene, a partial deletion or any other difference in the sequence that indicates a mutation in an essential region, or the lack of a DAP RNA and/or protein which may result in a loss of function may lead to a predisposition for cancer and/or metastasis. Preferably a battery of different DAP genes may be used, as well as different antibodies.

The DAP genes seem to play an important role in programmed cell death and the inhibition of their expression or neutralization of their expression products protects the cell from cytokine-promoted cell death. Examples of such genes are those whose sequences are depicted in Figs. 6, 8, 12, 15 and 16 or whose partial sequences are depicted in Fig. 13. The gene for the known protease cathepsin D, whose sequence is depicted in Fig. 14, is also revealed here for the first time as functioning as a DAP gene.

DAP DNA molecules useful in the death-promoting aspect of the invention may have the nucleic acid sequence of the DAP gene or other sequences which encode a product having a similar biological activity to that of the DAP product. Such DAP molecules include DNA molecules having a sequence other than that of the DAP gene but which, owing to the degenerative nature of the genetic code, encode the same protein or polypeptide as that encoded by the DAP gene.

It is well known that it is possible at times to modify a protein by replacing or deleting certain amino acids which are not essential for a certain biological function, or adding amino acids in a region which is not essential for the protein's biological function, without such modification essentially affecting the biological activity of the protein. Thus, a DAP DNA molecule useful in the death promoting aspect of the invention may also have a modified sequence encoding such a modified protein. The modified sequence has a sequence derived from that of the DAP gene or from that of the above degenerative sequence, in which one or more nucleic acid triplets (in the open reading frame of the sequence), has been added, deleted or replaced, with the protein product encoded thereby retaining the essential biological properties of the DAP product. Furthermore, it is known that at times, fragments of proteins retain the essential biological properties of the parent, unfragmented protein, and accordingly, a DAP DNA molecule useful in the death promoting aspect of the invention may also have a sequence encoding such fragments.

For example, the deduced amino acid structure of DAP-2 (DAP-kinase) suggests that this enzyme is a serine/threonine-type kinase. Its kinase domain was found to be composed of 11 subdomains typical of serine/threonine kinases, and is followed by a region that shares a significant homology with the calmodulin regulatory domains of other kinases. Adjacent to the latter, eight ankyrin repeats were found followed by two P-loop motifs. Moreover, a typical death domain module was identified at the 3' end of the protein, followed by a stretch of amino acids that is rich in serines and threonines (Feinstein, *et al.*, 1995, Trends Biochem. Sci. 20:342-44). The skilled artisan will know how to prepare active modified protein molecules and fragments on the basis of such information, and as further described below.

A DNA molecule useful in the death-preventing aspect of the invention may have a sequence which is an antisense sequence to that of the

DAP gene, or an antisense sequence to part of the DAP gene, blocking of which is sufficient to inhibit expression of the DAP gene. The part of the gene can be either the coding or the non-coding part of the DAP gene. The mRNA transcripts of the antisense sequences hybridize to the mRNA transcripts of the DAP gene and interfere with the final protein expression.

Non-limiting examples of cDNA clones containing specific antisense sequences are given in Table 1 below. Preferred antisense sequences are those sequences beginning at position 1000 and ending at position 1320 of the DAP-1 gene in Fig. 6, 3781-4148 of the DAP-2 gene in Fig. 8, 108-360 of the DAP-3 gene in Fig. 12, and 1203-1573 of the cathepsin D gene in Fig. 14.

Another DNA molecule useful in the death preventing aspect of the invention is a DNA molecule coding for a modified DAP product which is capable of inhibiting the activities of the unmodified DAP product in a dominant negative manner, such as a catalytically inactive kinase (DAP-kinase) or any other modified protein whose presence in the cell interferes with the normal activity of the native protein, for example by producing faulty hetero dimers comprised of modified and unmodified proteins which are inactive and the like. For example, a catalytically inactive DAP-kinase mutant which carries a lysine to alanine substitution within the kinase domain (K42A) was found not to be cytotoxic and protected cells from IFN-γ-induced cell death.

DNA molecules useful in the screening aspect of the invention comprise the sequence of a DAP gene or a sequence of a fragment thereof or specific antibodies.

In a first aspect, the present invention thus provides use of a therapeutically effective amount of an expression vector comprising a DNA sequence capable of inducing programmed cell death, in the preparation of a pharmaceutical composition for use in the treatment of a disease or a disorder

associated with metastasizing pathological cell growth, said DNA sequence being selected from the group consisting of:

(a) a DNA sequence expressed in cells, the expression product of which is involved in programmed cell death;

(b) a DNA sequence, other than the DNA defined under (a), which encodes the same expression product encoded by the DNA sequence defined in (a);

(c) a modified DNA sequence of (a) or (b) in which one or more nucleic acid triplets has been added, deleted, or replaced, the protein or polypeptide encoded by the modified DNA sequence mediating the programmed cell death similarly to the protein or polypeptide encoded by said gene as defined under (a) or (b); and

(d) fragments of any of the DNA sequences of (a), (b) or (c), encoding a protein or a polypeptide having said biological activity.

The term *"biological activity"* as used in this specification with respect to modified DNA or polypeptide molecules relates to the activity of the unmodified molecules with respect to the death-promoting, death preventing and screening aspects of the invention, as defined above.

In accordance with a specific embodiment, the present invention provides a use as described above, wherein said DNA sequence is a nucleic acid sequence expressed in cells, the expression product of which is involved in programmed cell death, being one of the following:

(i) a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 160-162 and ending at the triplet 466-468 of the sequence depicted in Fig. 6 (SEQ. ID. NO.:1);

(ii) a DNA sequence comprising a coding sequence beginning at nucleic acid triplet at position 287-289 and ending at a triplet at positions 816-818 of the sequence depicted in Fig. 6 (SEQ. ID. NO.:2);

(iii) a DNA sequence comprising a coding sequence beginning at nucleic acid triplet at position 337-339 and ending at the triplet at position 4603-4605 of the sequence depicted in Fig. 8 (SEQ. ID. NO.:3);

(iv) a DNA sequence comprising a coding sequence beginning at position 74-76 and ending at position 1268-1270 of the sequence depicted in Fig. 12 (SEQ. ID. NO.:4);

(v) a DNA sequence comprising a sequence depicted in Fig. 13 (SEQ. ID. NO.:5);

(vi) a DNA sequence of cathepsin depicted in Fig. 14 (SEQ. ID. NO.:7); or

(vii) a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 201-203 and ending at the triplet 3018-3020 of the sequence depicted in Fig. 15 (SEQ. ID. NO.:8).

A particularly interesting sub sequence of SEQ. ID. NO.:8 is a nucleic acid sequence beginning at position 1767 and ending at position 2529 of the sequence depicted in Fig. 15.

The use of the invention also relates to a a DNA molecule encoding the same protein or polypeptide encoded by any one of the nucleic acid sequences defined above, a nucleic acid sequence in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the sequence having essentially the same biological activity as that encoded by any one of the DNA molecules defined above, and a fragment of a nucleic acid sequence as defined above encoding a protein or polypeptide retaining a biological activity present in the protein or polypeptide encoded by said nucleic acid sequence as defined above.

The present invention also provides pharmaceutical compositions prepared according to the above use together with a pharmaceutically

acceptable excipient, and methods of treatment using the pharmaceutical compositions.

Also provided by the present invention is a method for choosing a chemotherapeutic treatment for a cancer patient comprising: determining whether the tumor cells of the patient comprise an active DAP gene; and choosing a chemotherapeutic drug whose mode of action induces apoptosis. DAP gene expression in tumor cells can increase the sensitivity of the cells to various chemotherapeutic drugs such as topoisomerase inhibitors (e.g. Adriamycin), mitotic inhibitors (e.g. Vincristine), glucocorticoids (e.g. Dexamethsone), folic acid antagonists (e.g. Methotrexate) and broad range protein kinase (PKC, PKA, etc.) inhibitors (e.g. Staurosporine).

In accordance with the prognostic aspect of the invention, there is provided a method for detecting the absence of a DAP gene, a partial deletion or a mutation (i.e. point mutation, deletion or any other mutation) in the DAP genes of an individual, or the absence of a DAP-related RNA or protein, comprising probing genomic DNA, cDNA or RNA from the individual with a DNA probe or a multitude of DNA probes having a complete or partial sequence of the DAP genes, or probing protein extracts with specific antibodies.

One example of a method in accordance with the prognostic aspect typically comprises the following steps:

(a) obtaining a sample from an individual suffering from said disease, said sample being a tissue section or either genomic DNA or mRNA obtained from cells, or cDNA produced from said mRNA;

(b) adding to said sample one or more nucleic acid probes, said one or more probes comprising a sequence selected from the group consisting of:

(i) a DNA sequence expressed in cells, the expression product of which is involved in programmed cell death;

(ii) a modified DNA sequence of (i) in which one or more nucleic acid triplets has been added, deleted, or replaced, said modified DNA sequence retaining the capability of hybridizing with the sequence of (i);

(iii) a sequence which is an antisense to the entire or part of the DNA sequence of (i) or (ii); and

(iv) an RNA sequence which is complementary to the DNA sequence of (i), (ii) or (iii) ;

(c) providing conditions for hybridization between the one or more probes and said sample; and

(d) determining on the basis of said hybridization whether a gene involved in programmed cell death is associated with said metastatic disease, an absence of hybridization indicating a lack of said gene, and an abnormal hybridization indicating a possible inactivation of said gene.

Another embodiment of this aspect may include the following steps:

(a) obtaining a sample from an individual suffering from said disease, said sample being a cell extract or a tissue section;

(b) adding to said sample one or more specific antibodies capable of binding a protein encoded by the DNA as defined above;

(c) providing conditions for the binding of said proteins in said sample by said antibodies; and

(d) determining on the basis of the binding of said proteins whether a protein involved in programmed cell death is associated with said metastatic disease, a lack of binding indicating an absence of said protein, and an abnormal binding indicating a possible modification of said protein.

Other examples of the prognostic aspect of the invention are well known to the skilled artisan and include, but are not limited to, Northern blots, RNase protection assays and various PCR procedures.

A specific embodiment of the prognostic aspect of the invention involves use of a complete or partial sequence of that shown in Figs. 6, 8, 12, 13, 14 or 15.

The mutation in the DAP gene indicating a possible predisposition to metastasis can also be detected by the aid of appropriate antibodies which are able to distinguish between a mutated and non-functional and a normal functional DAP gene product. In addition, mutations that abolish protein translation or loss of RNA due to promoter inactivation can be detected with the aid of antibodies that are reacted with protein cell extracts. One example is described below with respect to the loss of DAP-kinase RNA and protein in B cell lymphoma and bladder carcinoma cell lines.

A second aspect of the invention relates to methods of treatment and pharmaceutical compositions related to non-cytokine induced programmed cell death.

Thus, this aspect of the invention provides the use of a therapeutically effective amount of an expression vector comprising a DNA sequence capable of promoting non-cytokine-induced programmed cell death, in the preparation of a pharmaceutical composition useful in the treatment of a disease or a disorder associated with uncontrolled pathological cell growth, said DNA sequence being selected from the group consisting of:

(a) a DNA sequence expressed in cells, the expression product of which is involved in non-cytokine induced programmed cell death;

(b) a DNA sequence, other than the DNA defined under (a), which encodes the same expression product encoded by the DNA sequence defined in (a);

(c) a modified DNA sequence of (a) or (b) in which one or more nucleic acid triplets has been added, deleted, or replaced, the protein or polypeptide encoded by the modified DNA sequence mediating the programmed cell death similarly to the protein or polypeptide encoded by said gene as defined under (a) or (b); and

(d) fragments of any of the DNA sequences of (a), (b) or (c), encoding a protein or a polypeptide having said biological activity.

In accordance with a specific embodiment, the present invention provides a use as described above, wherein said DNA sequence is a nucleic acid sequence expressed in cells, the expression product of which is involved in programmed cell death, being one of the following:

(i) a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 160-162 and ending at the triplet 466-468 of the sequence depicted in Fig. 6 (SEQ. ID. NO.:1);

(ii) a DNA sequence comprising a coding sequence beginning at nucleic acid triplet at position 287-289 and ending at a triplet at positions 816-818 of the sequence depicted in Fig. 6 (SEQ. ID. NO.:2);

(iii) a DNA sequence comprising a coding sequence beginning at nucleic acid triplet at position 337-339 and ending at the triplet at position 4603-4605 of the sequence depicted in Fig. 8 (SEQ. ID. NO.:3);

(iv) a DNA sequence comprising a coding sequence beginning at position 74-76 and ending at position 1268-1270 of the sequence depicted in Fig. 12 (SEQ. ID. NO.:4);

(v) a DNA sequence comprising a sequence depicted in Fig. 13 (SEQ. ID. NO.:5);

(vi) a DNA sequence of cathepsin depicted in Fig. 14 (SEQ. ID. NO.:7); or

(vii) a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 201-203 and ending at the triplet 3018-3020 of the sequence depicted in Fig. 15 (SEQ. ID. NO.:8).

Another aspect of the invention relates to the use of a therapeutically effective amount of an expression vector comprising a DNA sequence capable of inhibiting non-cytokine induced programmed cell death, in the preparation of a pharmaceutical composition useful in the treatment of a disease or a disorder associated with non-cytokine induced programmed cell death, said DNA sequence being selected from the group consisting of:

(a) a sequence which is an antisense to the entire or part of the a DNA molecule expressed in cells, the expression product of which is involved in non-cytokine induced programmed cell death, said antisense being capable of inhibiting the expression of said DNA molecule; and

(b) a modified DNA sequence of a DNA molecule as defined in (i) in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the modified sequence having dominant negative effect manifested by the ability of said protein or polypeptide to inhibit said programmed cell death; and

(c) an inhibitor or antagonist of any of the proteins or polypeptides encoded by the DNA sequences defined above.

The prognosis aspect described above with respect to the first aspect of the invention is also relevant to the second aspect of the invention.

Other aspects of the invention will become apparent from the description following.

DESCRIPTION OF THE DRAWINGS

The present invention will be better understood from the following detailed description of preferred embodiments, taken in conjunction with the following drawings in which:

Figs. 1 A-D show RNA and protein expression of the DAP-1 gene, wherein:

Fig. 1(A) shows a Northern blot analysis of sense and antisense mRNA obtained from HeLa cells transfected with the constructs 230, 255, 260, 259 and control cells (parental cells) and probed by labeled cDNA fragments from construct 230. Total RNA was prepared from HeLa cells either before (parental) or after transfection with pTKO1 constructs #230 or #255 (group 1), #260 (group 5) and #259 (group 3) designated 230-t1, 255-t1, 260-t1 and 259-t1, respectively. Twenty g RNA were processed on Northern blots and DNA fragment #230 was used as a probe. The arrows point to the position of sense and antisense RNAs.

Fig. 1(B) shows a Northern blot analysis of sense and antisense mRNA obtained from HeLa cells transfected with control construct (DHFR-t2), 230 construct or control cells (parental) cells treated with (+) or without (-) 750 U/ml of IFN-γ for 24 h. The RNA was extracted from the indicated HeLa cells which were grown for 4 days in the absence (-) or presence (+) of IFN-γ (750 U/ml). The Northern blot containing 20 μg RNA samples was hybridized with the cDNA insert of λ1 phage. The Ethidium Bromide staining of the mRNA samples is shown.

Fig. 1(C) shows an SDS polyacrylamide electrophoresis gel of the expressed protein product of DAP-1 cDNA translated *in vitro* in a reticulocyte lysate preparation. *In vitro* translation of RNA (0.5 g) transcribed from the 1 cDNA (lane 2) and from the subclones p6, p4, p5 and p8 are shown in lanes 3-6, respectively. Lane 1 corresponds to the background obtained in the absence of RNA administration to the reticulocyte lysates. The labeled proteins were fractionated on 12% SDS polyacrylamide gels. The position of the radioactive molecular weight markers (Amersham) is marked. The two translated proteins, the major 15kDa and minor 22kDa proteins, are indicated by arrows.

Fig. 1(D) shows an immunoblot analysis of recombinant and cellular 15kDa DAP-1 protein. Bacterially produced DAP-1 protein (300 ng) and the indicated HeLa cell extracts (350 μg) were fractionated on SDS polyacrylamide gels (12%), blotted to nitrocellulose and reacted with affinity purified antibodies generated against the 15kDa DAP-1. The cells were treated with IFN-γ (750 U/ml) for 4 days before their extraction. The two arrows point to the position of the cellular DAP-1 protein. The antibodies also recognize two non-relevant bands of 60 and 45 kDa that are not modulated by the antisense RNA expression. Quantitation of the reduction in DAP-1 protein was done by densitometric analysis. The calibration of the protein content in each slot was done by referring to the signals of the non-relevant bands. The prestained protein markers (Sigma) are marked.

Figs. 2 A-D show RNA and protein expression of the DAP-2 gene, wherein:

Fig. 2(A) shows a Northern blot analysis of sense and antisense mRNA obtained from two clones of HeLa cells transfected with the control constructs (DHFR-t1 and DHFR-t2) and two clones of cells transfected with the 256 construct (t1 and t2). Total RNA was prepared from the 256-t1 and 256-t2 HeLa cell transfectants either before (0 hours) or at 3 and 24 hours after treatment with IFN-γ (750 U/ml) and 20 g samples were processed on Northern blots. Fragment #256 was used as a probe. The position of the sense and antisense mRNAs is indicated. The GAPDH mRNA levels were used for the calibration of the RNA amounts in each blot.

In **Fig. 2(B)** the blot consists of total RNA (20 μg) from K562 cells, parental HeLa cells, the two DHFR-transfected HeLa cell populations and the two HeLa cell populations that were transfected with the pTKO1-256. The blot was hybridized with the cDNA insert of λ29. The Ethidium Bromide staining of the RNA samples is shown.

Fig. 2(C) shows an *in vitro* phosphorylation assay. Cell lysates were prepared from COS-7 cells either before (lane 1) or after transfection with the PECE-FLAG expression vector that carries the coding region of the λ29 cDNA (lane 2). Samples of 400 μg were immunoprecipitated with anti-FLAG™ (M2) monoclonal antibodies (IBI) and subjected to phosphorylation assays.

Fig. 2(D) shows immunoblot analysis of recombinant and cellular DAP-2 protein. The COS-7 cells were transiently transfected with the PECE-FLAG-DAP-2 expression vector. Samples of cell lysates, 100 μg from COS-7 cells and 400 μg from HeLa cells, were fractionated on SDS polyacrylamide gels (7.5%), immunoblotted and reacted with affinity purified polyclonal antibodies raised against the N-terminal DAP-2 peptide. In the lower panel the blot was reacted with monoclonal antibodies against vinculin (Sigma Immunochemicals). Lanes: 1, non-transfected COS-1 cells; 2, transfected COS-1 cells; 3, DHFR-t1 cells; 4, 256-t1 cells; 5, 256-t2 cells. In lane 2 the same 160 kDa protein was also detected with anti-FLAG™ (M2) monoclonal antibodies (IBI) (not shown).

Figs. 3 A-C show morphological features of the cytostatic and cytotoxic responses to IFN-γ in HeLa cells. All cultures were seeded at an initial density of 10,000 cells per cm^2.

Fig. 3(A) shows light microscopy of HeLa cells transfected with pTKO1-DHFR construct (DHFR-t1 cells), on days 3 and 8 of culturing in the absence (a,c) or the presence (b,d) of IFN-γ (750 U/ml). (Magnification x 400). Note the absence of refractile mitotic cells during the cytostatic phase of responses to IFN-γ (in b) and the appearance of round cells that were detached from the substratum during the killing phase (in d).

Fig. 3(B) shows staining of DNA with DAPI; a. DHFR-t1 non-treated cells removed by trypsinization and mounted on glass slides. b. Detached

DHFR-t1 cells collected 7 days after IFN-γ treatment. Nuclei with condensed or fragmented chromatin are indicated by arrows. (Magnification x 1000).

Fig. 3(C) shows scanning and transmission electron micrographs of cells transfected with the control construct DHFR-t1 and the 230-t1 construct. DHFR-t1 HeLa cell populations (a-d) and the 230-t1 antisense transfected cells (e and f), were cultured either in the absence (a, c, e) or in the presence (b, d, f) of IFN-γ (750 U/ml). (a,b,e,f), scanning electron micrographs were taken after 7 days using GSM 6400 SEM (Jeol). Bars=10 mm (x2200 magnitude in all the four samples). (c and d), transmission electron micrographs taken after 7 days using TEM (Philips 410) at a magnitude of x2800. The condensed nuclei and the surface blebs are indicated by arrows.

Figs. 4 A-C show that the antisense RNA expression from plasmids of groups 1 and 2 reduces the susceptibility of HeLa cells to the killing effects of IFN-γ but has no effect on early IFN-γ signalling.

Figs. 4 (A-B) show the number of viable cells as determined by light absorption at 540 nm, as a function of time; the cells being transfected either with the control construct DHFR-t1 (• - 1(A) and 1(B)); the 255 or 230 construct (▲ - 1(A)) or with two clones t1 and t2 of the 256 construct (▲ - 1(B)). The results are shown both for cell growth with (+) and without (-) administration of 750 U/ml of IFN-γ. Each point is the average of a quadruplicate determination with a SD that ranged between 2-5%.

Fig. 4(C) shows a Northern blot analysis of 2-5A synthetase gene induction. The indicated HeLa cell transfectants were incubated for 24 hours in the presence (+) or absence (-) of IFN-γ (750 U/ml). Twenty mg of total RNA were analyzed. The cDNA of the 2-5A synthetase was used as probe.

Fig. 5 shows the restriction map of the λ1 cDNA clone that carries the DAP-1 cDNA.

Fig. 6 shows the DNA sequence and predicted amino acid sequence of DAP-1.

Fig. 7 shows the restriction map of the λ29 cDNA clone, that carries the DAP-2 cDNA.

Fig. 8 shows the DNA sequence and predicted amino acid sequence of DAP-2.

Figs. 9 A-C show DAP-2 sequence homologies to other serine/-threonine kinases and alignment of the ankyrin repeats of DAP-2, wherein:

In **Fig. 9(A)** the protein kinase domain sequences of the DAP-2 are aligned with the corresponding domains of other calmodulin-dependent kinases. The kinase subdomain structure (numbered I-XI) and the region implicated in calmodulin recognition and binding (designated as calmodulin regulatory region) are indicated. The obligatory conserved amino acids within the kinase domain are labeled with asterisks. Numbers at the right mark positions relative to the N-terminus of primary translational products of each kinase. Solid background indicates identical amino acids within the compared kinases. Stippled background indicates positions where the amino acids are not identical but similar. nm-mlck - non-muscle myosin light chain kinase (chicken); sm-mlck - smooth muscle myosin light chain kinase (chicken); skm-mlck - skeletal muscle myosin light chain kinase (rat); camdk-alph,-beta,-gamm -calcium/calmodulin dependent protein kinase II - α-, β- and γ- subunits, respectively; mlck-dicdi - dictyostelium discoidium (slime mold) myosin light chain kinase.

Fig. 9(B) shows alignment of kinase subdomains II and III of DAP-2 and the corresponding domains of different cell cycle dependent kinases. dm2 - Drosophila CDC2 homologue; pssalre - Human serine/threonine kinase PSSALRE; kpt2 - Human serine/threonine protein kinase PCTAIRE-2; kin28 - yeast (*S.cerevisiae*) putative protein kinase; mo15 - Xenopus protein kinase related to cdc2 that is a negative regulator of meiotic maturation; kkialre - human serine/threonine protein kinase KKIALRE.

Fig. 9(C) shows alignment of DAP-2 ankyrin repeats. Solid background indicates identical amino acids. A consensus sequence of the DAP-2 ankyrin repeats is shown at the bottom. The position of each individual repeat along the cDNA is illustrated in Fig. 9(B). ar 1-8, ankyrin repeats.

Fig. 10 shows Northern blot analysis of mRNA obtained from several hematopoietic cells probed with labeled DAP-1 cDNA.

Fig. 11 shows Northern blot analysis of mRNA obtained from liver, spleen or brain of normal embryos (2) and embryos with Down Syndrome (1) both probed with the labeled cDNA or DAP-1 or DAP-2. In order to evaluate levels of total mRNA, GAPDH was used (bottom).

Fig. 12 shows the DNA sequence and predicted amino acid sequence of DAP-3.

Fig. 13 shows a partial DNA sequence of DAP-4.

Fig. 14 shows the DNA sequence and amino acid sequence of cathepsin D.

Fig. 15 shows the DNA sequence and amino acid sequence of DAP-5.

Fig. 16 shows an immunoblot analysis of DAP-kinase expression. Subconfluent cultures of parental D122 cells, and of the different G-418-resistant derivative clones transfected with the pcDNA control vector (-cont.) or with pcDNA-DAP-kinase (-DAPk) were lysed and processed (300 μg protein per sample) as detailed before (Deiss, L.P., et. al., 1995, Genes Dev. **9**:15). Immunoblots were reacted with anti-DAP-kinase monoclonal antibodies (Sigma) and with anti-vinculin antibodies (Sigma). The endogenous levels of DAP-kinase in A9-F cells were used as a positive reference.

Fig. 17 shows the *in vitro* kinase activity of the ectopically expressed DAP-kinase gene. Samples of 1000 μg of total cell extracts were immunoprecipitated by anti-FLAG antibodies and subjected to kinase assay (upper panel) using myosin light chain (MLC) protein (5 μg; Sigma) as

exogenous substrate. The lower panel shows the DAP-kinase protein levels upon incubation of the same blot with anti-DAP-kinase antibodies.

Figs. 18A & B are *in vitro* growth curves of the transfected D122 clones. The cells were cultured in 24-well plates at an initial cell density of $1x10^4$ cells per well; the medium was supplemented with either 10% (Fig. 18A) or 1% (Fig. 18B) fetal calf serum (FCS) (Gibco BRL). At 24 hours time intervals cell numbers were quantified by the crystal violet method (Kueng, W., et al., 1989, Anal Biochem. **182**:16) and the O.D. of lysed cells was measured at λ=540 nm. Data are mean of duplicate determinations of two experiments. Symbols:

(○) D122; (◊) 1-cont.; (□) 4-cont.; (X) 6-DAPk; (♦) 28-DAPk; (▽) 42-DAPk; (●) 48-DAPk.

Fig. 19 shows local tumor growth in footpads as a function of number of days post injection. The different D122-transfected clones were injected into the footpads of C57BL/6 mice (10-12 week old females). Diameters of tumor bearing feet were measured every 1-3 days. Values represent the mean pad diameter of the individuals in each group (8 per group). The symbols are as in Fig. 18.

The SD ranged between 0% to 32% of the measurements. An unpaired one-tailed student's t-test performed at numerous time points indicated that differences between sizes of the growing tumor formed by the slowest growing control clone (1-cont.) and these formed by the 28-DAPk or 42-DAPk clones were significant at $P<0.001$. It can be seen that transfection with DAP-kinase delays the growth of local tumors.

Fig. 20 shows average lung weight and mean number of metastatic lesions of intraveneous injected mice. Mice, as above, were injected in the tail vein and sacrificed 30-32 days later. Lungs were removed weighed and fixed in Bouin's solution. The number of metastatic nodules were determined by counting surface nodules under a binocular. Values are mean ± SD of 5

individuals in a group, presenting either lung weight (in bars) or number of metastatic nodules per mouse (in Table). The solid line in the bars graph indicates the average lung weight of non-injected mice.

Differences between the less aggressive 1-cont. clone and each one of the DAP-kinase transfectants were significant at $P<0.001$ for 48-, 28- and 42-DAPk clones and $0.025<P<0.05$ for the low expressing clone 6-DAPk (the latter clone differed from 4-cont. clone and parental D122 cells at $P<0.001$). Thus, transfection with DAP-kinase strongly suppressed experimental metastasis.

Fig. 21 are photographs of three representative lungs from each group of mice as in Fig. 20. Note the differences in lung size and surface nodules compared to lungs obtained after I.V. injections with the A9-F low metastatic clone (used as a reference). Scale bar, 1 cm.

Fig. 22 shows immunoblot analysis for DAP-kinase protein levels of clone 28-DAPk, as in Fig. 16. The expression was tested both in the original clone used for the I.F.P. and I.V. injections (lanes 1 and 3, respectively) and in tumor cells that were re-cultured from the lungs of injected mice. The latter cell cultures were recovered either from the multiple spontaneous lung nodules that appeared 35 days post surgery (lane 2) or from the very few nodules that appeared in the experimental metastasis assays (lane 4).

The DAP-kinase levels were below detection limits in the 4-cont.-transfected clone both before injections as well as after recovery of tumor cells from the spontaneous lung lesions (lanes 5 and 6, respectively), confirming that the tumor cells were not contaminated with surrounding DAP-kinase positive primary lung cells. Lane 7 displays the expression levels of endogenous DAP-kinase in the low metastatic clone A9-F.

Fig. 23 shows immunoblot analysis of clone 42-DAPk tested before and after its recovery in culture from the spontaneous lung metastases formed 34 days post foot amputations (lanes 1 and 2, respectively). Lane 3 shows the

expression levels of endogenous DAP-kinase in the low metastatic clone A9-F.

Fig. 24 shows an immunoblot analysis of cell cultures released from spontaneous lung metastatic lesions, formed after the I.F.P. injections of clone 28-DAPk. The cultures were treated *in vitro* with 10 μM 5-aza-2'-deoxycytidine for 24 hours. The non-treated and drug-treated cultures were tested for DAP-kinase protein expression either on day 3 (lanes 1 and 2, respectively) or on day 14 post treatment (lanes 3 and 4, respectively).

Fig. 25 shows in situ TUNEL staining of footpad sections prepared on day 5 after local injection of $2x10^5$ 4-cont. cells (left-hand photograph) or 42-DAPk cells (right-hand photograph). Peroxidase staining of fragmented DNA and counterstaining of the sections of methyl green dye were performed according to the manufacturer's instructions (ApopTag® Plus Peroxidase Kit; Oncor, Gaithersburg). Scale bar, 100 μm.

Fig. 26 shows DAPI staining of the nuclei before and after treatment with TNF-α. Exponentially growing cells corresponding to 18-cont. and 42-DAPk transfectants were treated with a combination of murine TNF-α (100 ng/ml; R&D systems, Minneapolis) and cycloheximide (5 μg/ml; Sigma) (right panels marked by +), or with cycloheximide alone (left panels marked by -). DAPI staining was performed after 6 hours. The arrows point to apoptotic nuclei.

Fig. 27 illustrates time kinetics of killing by TNF-α. The conditions of treatment with TNF-α and cycloheximide and assessment of apoptotic nuclei by DAPI staining were as in Fig. 26. The 42-DAPk transfectants (X) were compared in this assay to the parental D122 cells (O), to 4-cont. (□) and to 18-cont. (◊). The values are the mean of percent of intact nuclei ±SD counted by scoring 5 different fields, 100 total nuclei in each field, at the indicated time points.

The differences between 42-DAPk and 4-cont. clones were significant at $P<<0.001$ both at the 4.5 and 6 hours time points, and at $0.005<P<0.001$ with respect to the 7 hours time point.

Fig. 28 shows the results of assays of response to TNF-α and cycloheximide as described in Figs. 26 and 27. The original 42-DAPk clone was compared to the cultures recovered from the spontaneous lung metastases described in Fig. 23 (named here 42-DAPk*). Values are mean of percent apoptotic nuclei ± SD counted by scoring 5 different fields, 100 total nuclei in each field at 6 hours after exposure to the double treatment.

The results show that *in vivo* selection for attenuated DAP-kinase expression ablates the increased sensitivity of clone 42-DAPk to TNF-α. **Fig. 29** shows the growth of the D122-transfectants in a semi-solid medium under anchorage-independent conditions. The different clones were cultured in 0.33% soft agar (Bacto-agar; Difco) at an initial cell number of $5x10^3$ cells per 6 cm plate, on top of a layer containing 0.5% agar. The diameters of the clones that appeared on day 7 were measured under a light microscope. Values are the mean colony diameter of 100 clones from each group±SD.

Clones 1-DAPk and 21-DAPk expressed exogenous DAP-kinase protein at levels which were comparable to clone 28-DAPk (Fig. 16). The difference between the controls (e.g., 18-cont.) and the DAP-kinase-transfectants (e.g., 1-DAPk) was significant at $P<<0.001$.

Fig. 30 shows microscopy of the clones cultured in soft agar for seven days as in Fig. 29 , comparing the parental D122 cells (left: a,c) to DAPk-42 cells (right: b,d). The bars correspond to 350 μm in the upper panels (a,b) and to 80 μm in the lower panels (c,d).

Fig. 31 is a schematic representation of DAP-kinase and its mutants used in these studies. The various motifs and domains as predicted by the deduced amino acid sequence and/or experimental work are shown. The

numbers below indicate the amino acid positions. The K42A, CaM and (1-1271) DD mutants are presented schematically below.

Fig. 32 shows *in-vitro* kinase activity of the DAP-kinase. DAP-kinase or DAP-kinase mutant proteins were assayed *in-vitro* for kinase activity in the presence of Ca^{2+} / CaM and MLC as described below. The proteins were run on 11% SDS-PAGE and blotted to nitrocellulose membrane. The upper and the middle panels show the autophosphorylation of DAP-kinase and MLC phosphorylation, respectively, as seen after exposure to X-ray film. The lower panel shows the DAP-kinase proteins by incubation of the same blot with anti-FLAG antibodies and ECL detection.

Fig. 33A-C show that DAP-kinase binds calmodulin and its activity is regulated by calcium/calmodulin.

(33A). Calmodulin overlay on DAP-kinase. The upper panel displays the results of hybridization with ^{35}S-met labeled recombinant CaM. The lower panel shows the results of hybridization of the same blot with anti-FLAG antibodies to detect the DAP-kinase protein.

(33B). Ca^{2+} / CaM regulation of DAP-kinase activity. DAP-kinase was subjected to *in-vitro* kinase assay as described below, in the presence or absence of Ca^{2+} and CaM (reaction was stopped after 15 min. for detecting the autophosphorylation, or after 2 min. to measure MLC phosphorylation). The lower panels shows the results of incubation with anti-FLAG antibodies (in the same blot).

(33C). DAP-kinase DCaM activity is maximal in the absence of Ca^{2+} CaM. Details are as in (33B).

Fig. 34A & B illustrate how ectopic expression of DAP-kinase induces cell death.

(34A). HeLa cells ($5x10^5$ cells/plate) were transfected with 20 μg DNA of pcDNA3 vector or with DAP-kinase constructs cloned into the same vector. After 48 hours, the cell cultures were split 1:5 and subjected to

selection with G-418. After 2-3 weeks the plates were stained with crystal-violet.

(34B). HeLa cells ($5x10^5$ cells/plate) were transfected with 20 μg DNA of the empty pSBC-bl plasmid, or with the vector which carries the wild-type DAP-kinase. The cells were grown for 48 hours in the absence of tetracycline and stained with X-Gal solution. The frequency of blue cells with an apoptotic rounded morphology was assessed by counting 600 total blue cells from 6 different fields coming from duplicate transfections. The arrow points to a transfected dying cell.

Fig. 35A & B illustrate how a DAP-kinase K42A mutant protects HeLa cells from the IFN-γ-induced cell death.

(35A). HeLa cells ($5x10^5$ cells/plate) were transfected with 20 μg DNA of empty pcDNA3 vector or DAP-kinase-K42A cloned into the same vector. After 48 hours, the cells were split 1:5 and subjected to selection with G- 418 and 200U / ml of IFN-γ. After 2-3 weeks of selection the plates were stained with crystal-violet. Pictures were taken under light microscopy using Kodak TMXIOO film (magnification x40).

(35B). Number of surviving colonies per $1cm^2$ was counted and normalized according to the number of colonies appearing in G-418 selections which were performed in the absence of IFN-γ. Values represent the average of ten representative fields.

Fig. 36A-D illustrate analysis of DAP-kinase expression in various hematopoietic cell lines.

36A and B: Northern blot analysis of polyA+ RNA from various cell lines using probes for DAP-kinase and c-Abl, respectively.

36C and D: Western blot analysis of DAP-kinase protein and vinculin (as an unrelated protein reference), respectively.

Fig. 37 shows Western blot analysis of DAP-kinase in the bladder carcinoma cell lines T24 and HT1376 treated with 5-azadeoxycytidine.

Protein extracts were loaded as follows: Lane 1 and 4- T24 and HT1376 bladder carcinoma cells non-treated, respectively; lanes 2 and 5- T24 and HT1376 bladder carcinoma cells, treated with 5-azadeoxycytidine and collected after 2 passages without treatment, respectively. Lane 3- T24 bladder carcinoma cells collected after 6 passages without treatment. The same blots were reacted with anti-vinculin and anti-DAP3 antibodies.

Fig. 38A-C show that the DAP-5 763bp fragment is expressed in HeLa cells at very low levels, as compared to subgroup I cDNA fragment.

38A. Northern blot analysis of RNA from pTKO1-260 or pTKO1--DHFR transfected cells. RNA was extracted from the indicated HeLa cells. The Northern blot containing 20 μg of total RNA samples was hybridized with the DAP-5 763bp fragment(#260). 1. DHFR-t1; 2. 260-t1; 3. 260-t2.

38B. IFN-γ resistant phenotype of HeLa cells transfected with pTKO1--260. HeLa cells were transfected with either control vector pTKO1-DHFR or with the isolated pTKO1-260. Pools of more than 10^4 independent clones were first selected with hygromycin B to generate polyclonal populations of stably transfected cells. These pools were plated in 9cm plates (100,000 cells per plate) and double selected with IFN-γ (1000 units/ml) and hygromycin B (200 g/ml). After 4 weeks of selection, the cells were stained with crystal-violet. In the absence of IFN-γ these plates reach confluency after 4 days.

38C. Comparison of the expression levels of RNAs from subgroup I and II. 260-t1 and 260-t2 represent the same extracts used in 38A. The Northern blot containing 20 μg of RNA samples was hybridized with the BglII- BamHI fragment containing the SV-40 splice and polyadenylation signal (Deiss & Kimchi, 1991) which is part of the SV-40 promoter driven, #260 containing, mRNA expressed from the episome. D. Same as in 5C but hybridized with a probe recognizing the hygromycin B resistance gene driven by the TK promoter.

Fig. 39 shows β-galactosidase activity assays of HeLa-tTA cells transfected with the DAP-5 763bp fragment or its mutated versions.

Stable polyclonal populations transfected with the pSBc-bl vector (control), with the pSBc-bl-260 (260), or with the pSBc-bl vector harboring either the single or the triple ATG #260 mutant were established by selection with 10 μg/ml bleomycin. After two weeks the drug was removed and cultures were further expanded. Growing cells were fixed with 3% paraformaldehyde for 5 minutes, rinsed twice with PBS and checked for β-galactosidase activity using the X-gal as a substrate. Photography was done under phase microscopy using Kodak Ectachrome 160T.

Fig. 40A & B show *in vitro* translation of the DAP-5 763bp fragment and immunoblot analysis of the mini-protein in cells expressing the #260 fragment.

40A. *In vitro* translation of RNA transcribed from Bluescript vector harboring various DAP-5 versions was done in rabbit reticulocyte lysates.The resulting ^{35}S labeled proteins were fractionated on 12.5% SDS-PAGE. The position of radioactive molecular mass markers (Amersham) are marked. 1. non-programmed rabbit reticulocyte lysates; 2. Full length DAP-5 3.8kb clone; 3. DAP-5 763bp fragment (#260); 4. mutated #260 fragment: ATG at position 1785 converted to AAG (single ATG mutant); 5. mutated #260 fragment: ATG at position 2010 was converted to TTC and ATG at position 2040 to ATC; 6. triple ATG mutant harboring all the above mentioned mutations. The position of the translated mini-protein is marked by an arrow; the additional higher bands are non-specific background that often appears also in non-programmed reticulocyte lysates.

40B. HeLa-tTA cells transfected with either the pSBc-bl vector or the pSBc-bl vector harboring the #260 fragment were lysed and fractionated on 10% SDS-PAGE, blotted onto nitrocellulose and reacted with affinity purified polyclonal antibodies (1:20 dilution) raised against a GST-fused recombinant

product. The arrow points the position of the DAP-5 mini-protein specific doublet that had an approximate size of 28kDa.

Figs. 41A, B, C & D show Involvement of cathepsin D protease in IFN-γ mediated cell death.

(41A). Protection from IFN-γ induced cell death by anti-sense RNA expression. **(a)** One of the DHFR-transfected polyclonal cell populations (squares) and of the anti-cath-D-transfected polyclonal cell populations (circles) were treated with IFN-γ (1000 U/ml; filled symbols) or left untreated (open symbols). Viable cells were stained with neutral-red and the dye uptake was measured at $\lambda_{540\ nm}$. Each point represents an average of a quadruplicate determination. **(b)** Two independent DHFR-transfected polyclonal cell populations (open and filled squares) and a pair of anti-cath-D-transfected polyclonal cell populations (open and filled circles) were treated with IFN-γ (1000 U/ml) or left untreated. Fraction of viable cells was determined by comparing neutral red dye uptake of IFN-γ treated cells to non-treated cultures at the indicated time points. Each point represents an average of a quadruplicate determination ± S.E.

(41B) Regrowth of viable cells after withdrawal of IFN-γ (1000 U/ml) from DHFR and anti-cath-D transfectants. Cells were seeded at an initial density of 10,000 cells/cm^2 , treated with a combination of hygromycin B and IFN-γ (1000 U/ml) for two weeks, washed and stained with crystal violet 7 days later.

(41C) Protection from IFN-γ-induced cell death by pepstatin A. The HeLa cells (DHFR and anti-cath-D transfectants), were incubated for 8 days with IFN-γ (1000 U/ml) either in the presence of pepstatin A (10^{-4}M in 0.2% DMSO) or in its absence (0.2% DMSO alone). The DHFR-transfected cells were also tested for responsiveness to pepstatin A in the absence of IFN-γ.

Data are given as mean neutral-red dye uptake from quadruplicate samples ± S.E.

(41D) Light microscopy of HeLa cells on day 8 of IFN-γ-treatment : (a) DHFR transfectants with no inhibitor; (b) anti-cath-D transfectants cultured in the presence of pepstatin A (Magnification, 200x).

Fig. 42A, B, C & D show regulation of expression and processing of cathepsin D protease by IFN-γ and TNF-α.

(42A & B) Immunoblot analysis of cathepsin D forms before and after treatment with IFN-γ (1000 U/ml). Cell lysates were prepared at the indicated time points from parental HeLa cells (**A**) and from DHFR and anti-cath-D transfectants (**B**). Samples of 300 μg were fractionated on SDS-polyacrylamide gels (12%) blotted to nitrocellulose, and detected using the ECL system (Amersham). The sizes of cathepsin D forms are shown. The same blots were reacted with polyclonal antibodies generated against the copper zinc superoxide dismutase (SOD) to correct for possible differences in protein amounts in each slot.

(42C) A scheme that depicts the different steps of cathepsin D processing as previously reported for rat cathepsin D (Fujita et al., 1991, BBRC 179:190-196).

(42D) Immunoblot analysis of cathepsin D forms before (lane 1) and after treatment of U937 with TNF-α (lanes 2 and 3; 24 and 48 hours, respectively).

Fig. 43A & B show involvement of cathepsin D protease in Fas/APO-1, and TNF-α mediated cell death.

(43A) Suppression of Fas/APO-1- mediated cell death by anti-cathepsin D RNA or by pepstatin A. The HeLa cells (DHFR and anti-cath-D transfectants; 20,000 cells per microtiter well), were exposed to anti-APO-1 antibodies for 40 hours as described below. Pepstatin A (10^{-4}M in 0.2% DMSO) was added where indicated to the DHFR transfectants 20

hours before their exposure to the anti-APO-1 antibodies. Viability was assessed by the neutral red assays in quadruplicate samples; results are expressed as percent of dye uptake at the end of each treatment out of the total uptake in the corresponding control wells, which were not exposed to the antibodies (100% viability).

(43B) Pepstatin A interferes with the TNF-α-induced apoptotic cell death in U937 cells. The cells were seeded at a density of $2x10^5$ cells /ml, 24 hours after their preincubation with pepstatin A (10^{-4}M in 1% DMSO) or with DMSO alone. Where indicated, TNF-α (100 U/ml; 10 ng/ml) was added and 6 hours later samples were cytospinned on glass slides and stained with DAPI (0.5 μg/ml, Sigma). Microscopy was performed under fluorescent light conditions (Magnification, 1000x). Nuclei with fragmented chromatin are indicated by arrows; empty arrowhead point to mitotic nuclei. Data are presented as the percentage of cells with a fragmented nuclear morphology ± S.E. For each condition a minimum of 400 cells in 14 separate fields were scored.

Fig. 44A, B, C & D show that ectopic expression of cathepsin D reduces cell viability.

(44A&B) X-Gal staining of HeLa cells co-transfected with lacZ (driven by CMV promoter) and either with the cathepsin D cDNA (driven by a tetracycline-repressible promoter) or with the control vector. In both cases cells were cultured in the absence of tetracycline and stained after 48 hours with X-Gal solution for 3 hours. Light microscopy micrographs are shown (magnification, 200x). Examples of normal blue-stained cells (in **A**) and of apoptotic blue-stained cells (in **B**) are indicated by arrows.

(44C) The frequency of blue cells with an apoptotic rounded morphology was assessed by counting 800 total blue cells from 8 different fields coming from duplicate transfections (described in **A** and **B**).

(44D) Assessment of secreted alkaline phosphatase (SEAP) in the growth medium of HeLa cells, co-transfected with SEAP and with either the control vector or the abovementioned cathepsin D vector; each transfection was divided into two plates, one of which was immediately supplemented with tetracycline (1.5 μg/ml). SEAP activity secreted into the growth medium during the last 5 h of incubation was determined 48 hours after transfections. Data of SEAP activity were obtained in duplicates from four experiments. The values give the percentage of SEAP activity measured in the absence of tetracycline out of total activity produced in the presence of tetracyline.

Fig. 45A & B show how DAP-kinase is localized to the cytoskeleton.

(45A). SV-80 cells transiently transfected with DAP-kinase-K42A were stained at 48 hours with anti-FLAG antibodies and fluoresceine-conjugated phalloidin as described below. Both pictures represent the same field (magnification x 400).

(45B). Detergent extraction of HeLa cells. HeLa cells were extracted with 0.5% triton X-100 to soluble fraction (Sol) and insoluble fraction (InSol) as described below. The protein extracts were separated on 10% SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was reacted with anti-DAP-kinase monoclonal antibodies, anti-tubulin antibodies and anti-actin antibodies as indicated.

Fig. 46A & B illustrate mapping of the region responsible for cytoskeletal binding.

(46A). Immunostaining of recombinant DAP-kinase. COS cells, transfected with pECE-FLAG - DAP-kinase, were immunostained with anti-FLAG monoclonal antibodies as as described below (Magnification x 400).

(46B). COS cells were transfected with pECE or pCDNA3 vectors carrying either DAP-kinase or DAP-kinase deletion mutants as indicated. The cells were extracted with 0.5% Triton X-100 as described in Fig. 45.

Detection was carried out with anti-FLAG antibodies. Sol - soluble fraction, InSol- detergent insoluble fraction. Schematic representation of DAP-kinase deletion mutants is shown at the left.

Fig. 47A & B show changes in actin cytoskeletal organization in the IFN-γ-induced cell death and upon ectopic expression of constitutive DAP-kinase.

(47A). HeLa cells, grown on glass coverslips, were treated with IFN-γ (1000 U/ml) (b) or were left untreated (a). After 4 days the cells were stained with fluorescein-conjugated phalloidin (magnification x 1000).

(47B). REF-52 cells were transiently transfected with DAP-kinase mutants as indicated. After 48 hours the cells were triple stained with anti-FLAG antibodies, fluorescein-conjugated phalloidin and DAPI as described below (magnification x 100). The arrows point to the transfected cells.

DETAILED DESCRIPTION OF THE INVENTION

I. Isolation of antisense cDNA's that protect cells from the cytotoxic effects of IFN-γ

(A) Experimental procedure

(A_1) Obtaining cDNA clones

A cDNA library (100 g DNA) was generated from a mixture of mRNA's harvested before and at 1, 2, 4, 12, 24 and 48 hours after treatment of HeLa cells with IFN-γ (200 U/ml). It was cloned in antisense orientation into the EBV-based pTKO1 expression vector, as previously described in detail (Deiss and Kimchi, *supra*). The resulting expression library of about 10^5 independent clones was introduced into $8X10^6$ HeLa cells (10^6 cells per 9 cm plate) by the calcium phosphate transfection technique. In order to determine the efficacy of transfection, a fraction of the transfectants was selected with hygromycin B (200 μg/ml, Calbiochem). The resulted efficacy was around

5%. In parallel, the majority of the transfected cells were plated at a cell density of 1500 cells per cm^2 and were selected with both hygromycin B (200 μg/ml) and IFN-γ (750 U/ml). Selective media was changed every 3-4 days. After 28 days the cells that survived and/or grew in the presence of IFN-γ were expanded for 2 weeks and pooled. The extrachromosal DNA was obtained according to the method of Hirt (Hirt, B. (1967) *J. Mol. Biol.*, **26**:365), cleaved with the restriction enzyme DpnI and introduced into *Escherichia coli* HB101 host cells. The cleavage with DpnI ensured that only episomal DNA that have replicated in HeLa cells was transfected into bacteria.

A few bacterial clones were obtained by the above procedure which included DNA antisense sequences, some of which were able to protect the cells from the death-promoting effects of IFN-γ.

(A_2) Classification of the antisense cDNA clones

Plasmid DNAs were prepared from 10 individual bacterial clones. PCR amplified cDNA inserts were generated from each plasmid using specific primers that correspond to the immediate flanking sequence of the cDNA insertion sites in the pTKO1 vector. The size of the cDNA inserts ranged between 300 to 800 bp. The PCR fragments were used as labeled probes to search on Southern blots for possible cross hybridization between some of the rescued antisense cDNA clones.

(B) Results

(B_1) Classification of Clones

The above 10 cDNA clones were classified into six distinct non-overlapping groups, some constituting several members (clones) and some constituting of a single member. Those clones relevant for the present invention are shown in the following Table 1:

Table 1:Initial characterization of antisense cDNA clones rescued from IFN-γ-treated HeLa cells

No.	Antisense cDNA clones	cDNA length (bp)	mRNA size (Kb)	DNA product
1.	230, 254, 255, 264, 258	320	2.4	DAP-1
2.	256	367	6,3	DAP-2 (kinase)
3.	259	252	1.7	DAP-3
4.	253	200	4.5	DAP-4
5.	260	763	3.8	DAP-5

Inserts 230, 254, 255, 264 and 258 of group 1 seemed to be completely identical to one another. The PCR fragments were sequenced and the results were compared with sequences present in the EMBA nucleic acid database. All inserts of groups 1 through 5 were found to be novel.

(B_2) Detection of mRNA

The DNA fragments thus obtained were used to detect and determine the expression level in HeLa cells of mRNA which hybridized to these fragments. 20 μg of total RNA from the parental HeLa cells were fractionated on gels, blotted and reacted with the different probes. Each probe recognized a single mRNA transcript of a different size (Table 1). Expression levels of mRNA's reactive with group 2 were low while those reactive with group 1 were relatively high.

II. Second transfection by isolated antisense cDNA

Levels of expression of antisense RNA in secondary transfectants

(A) Experimental procedure

To ensure that the above isolated antisense cDNA's are sufficient in order to protect cells from the death promoting effects of IFN–γ, subconfluent monolayers of HeLa cells were transfected with 40 μg DNA of the individual rescued pTKO1 plasmids (in duplicates) and subjected to the single selection of hygromycin B. Pools of approximately 10^4 hygromycin resistant clones were generated from each transfection and were kept as 6 duplicates of stable polyclonal populations. The sensitivities of the above clones to an application of IFN-γ was then determined.

The vector pTKO1-DHFR (Deiss and Kimchi, *supra*) which carried a non-relevant construct served as control. The control vector was introduced in parallel into HeLa cells and produced two independent polyclonal population of stable transfectants designated DHFR-t1 and t2.

The double stranded cDNA fragments from construct 230 and 256 (from groups 1 and 2, respectively) were used as probes in Northern blot analysis in order to detect mRNA transcripts both in non-transfected and transfected HeLa cells. These two specific cDNA inserts were labelled by commonly used commercial labelling kits. They were subcloned into Bluescript™ vectors (Stratagene, USA) to facilitate both the preparation of the cDNA inserts and the production of single stranded RNA probes therefrom.

(B) Results

Constructs 230 (group 1)

As can be seen in Fig. 1A the cDNA insert in this construct hybridized to a single endogenous 2.4 Kb mRNA transcript, both in non-transfected and transfected HeLA cells. In stable transfectants containing the antisense constructs of clones 230 and 255, an additional composite antisense transcript was detected by this 230 probe. It consisted of 320 bases of the original

cDNA insert and 800 additional bases of sequences derived from the expression cassette (SV40 early promoter together with sequences till the polyadenylation signal). One of the RNA labeled strands produced by the Bluescript™ vector hybridized exclusively to the endogenous 2.4 Kb mRNA while the complementary strand hybridized only to the 1.1 Kb RNA confirming that the latter is indeed an antisense mRNA (data not shown).

The amount of the antisense RNA in clones 230 and 255 exceeded the sense mRNA levels by 3 to 6 fold (Figs. 1A, 1B). After IFN-γ treatment the level of antisense expression was further elevated due to the presence of IFN-γ-stimulated response element (ISRE) in the pTKO1 vector (Deiss and Kimchi, *supra*), thus leading to 15 fold excess of antisense over sense transcripts (Fig. 1B). The endogenous 2.4 Kb mRNA level was neither modulated by IFN-γ, nor influenced by the high antisense expression.

Construct 256 (group 2)

As can be seen in Figs. 2A and 2B, the construct of the 256 clone (367 bp in size) hybridized on Northern blots to a single endogenous 6.3 Kb mRNA transcript which was expressed in all tested cells at relatively low levels. In the 256-t1 and t2 transfected cells it also hybridized to a composite 1.2 Kb RNA that consisted of 367 bases of the cDNA insert and 800 bases of sequences derived from the expression cassette in the vector (Fig. 2). The antisense orientation of fragment #256 in the pTKO1 vector was confirmed upon sequencing of the sense cDNA clone (Fig. 7). The amount of the antisense RNA expressed from pTKO-1 plasmid #256 in non-treated HeLa cells exceeded the sense mRNA levels by more than 100 fold. Moreover, due to the presence of IFN-stimulated response element (ISRE) in the pTKO1 vector, the levels of antisense mRNA expression were further elevated after IFN-γ treatment (Fig. 3).

III. Response of cells transfected with antisense cDNAs to IFN-γ

(A) Experimental procedure

The HeLa polyclonal population transfected with the individual antisense cDNAs were cultured in the presence of both hygromycin B and IFN-γ (750 U/ml). Growth and viability parameters were examined: (1) under the light microscope, (2) by electron microscopy, and (3) by DAPI staining (0.5 μg/ml; Sigma). For more detailed quantitation, a neutral red uptake assay was performed: the different polyclonal HeLa cell populations were cultivated in 96-well microtiter plates at subconfluent cell densities and then treated with IFN-γ (750 U/ml) or left untreated. All the cells were continuously maintained in a hygromycin B-containing medium to select for transfected cells. The two DHFR-transfected HeLa cell populations (t1, t2), prepared as described above, served as control cultures that display the typical growth sensitivity curves to IFN-γ. The examined antisense cDNA transfected cells were the 230-t1, 255-t1 (group 1) and 256-t1, 256-t2 (group 2). Viable cells were stained with neutral-red and the dye uptake was quantified by measuring O.D. at 540 nm in quadruplicates during the 14 days of the experiment.

(B) Results

The microscopic examination of parental and control DHFR-transfected HeLa cells revealed that IFN-γ triggered a biphasic pattern of responses. The cells stopped proliferating during the first four days of IFN-γ treatment but still remained viable (in trypan-blue exclusion tests) and displayed a flattened morphology characteristic of the cytostatic responses to IFN-γ (Fig. 3A, b). The reduction in the proliferation rate during this period was also measured by a sharp decline (by more than 90%) in the thymidine uptake into DNA (not shown). This type of IFN-γ-induced proliferation arrest was then followed by massive cell death that occurred in a non-synchronous fashion over a period of an additional 10 days. The cells gradually reduced their size, rounded up and detached from the plates (Figs. 3A, d). Staining of

DNA with DAPI after detachment of cells from the substratum revealed gross changes in the nuclear morphology characteristic of programmed cell death. This included nuclear pyknosis, chromatin condensation, sometimes detected preferentially at the nuclear periphery, and chromatin segmentation (Fig. 3B, b). Transmission electron micrographs of the IFN-γ-treated cells prior to their detachment revealed other morphological changes including the disappearance of surface microvilli, surface blebbing, budding off cytoplasmic projections and cytoplasmic disintegration, in addition to the nuclear pyknosis and chromatin condensation (details shown in Fig. 3C, d). The antisense RNA expression from pTKO-1 plasmid of group 1 reduced the susceptibility of the cells to the killing effects of IFN-γ: more cells survived on the plates and the above-mentioned death associated morphological changes appeared at much lower frequency (compare the scanning electron micrographs of the IFN-γ-treated DHFR-transfected cells in Fig. 3C, b to the IFN-γ-treated 230-t1 cells in Fig. 3C, f). Similar microscopic observations, showing protection from the IFN-γ-induced cell death, were also made with respect to three other clones from the aforementioned groups of antisense cDNAs, i.e. 2-6 (see below).

A neutral-red uptake assay was then performed to determine more accurately, on a quantitative basis, both the typical biphasic responses of control cultures to IFN-γ and the reduced susceptibility of the antisense expressing cultures to the IFN-γ-induced cell death. The two DHFR-transfected HeLa cell populations (t1, t2) served as the control cultures in this assay and the antisense cDNA transfected cells examined were the 230-t1, 255-t1 (group 1) (Fig. 4A) and 256-t1, 256-t2 (group 2) (Fig. 4B). In the absence of IFN-γ, all the transfected HeLa cells behaved the same and displayed practically identical growth curves suggesting that the antisense RNA expression had no effects on the normal growth of cells. Another feature that was not changed by the antisense RNA expression was the extent of the

cytostatic responses to IFN-γ. As shown in Figs. 4A and 4B, IFN-γ has similarly reduced the proliferation rate of all the transfected cultures and they all displayed the same extent of reduction in the neutral-red dye uptake during the first 4 days (before cell death starts to be microscopically evident). After 4 days of treatment the picture changed drastically. While almost all control cells died during the subsequent days of IFN-γ treatment leading to minimal values of the neutral-red dye uptake on day 14, a significant fraction of cells that expressed antisense RNA survived in the presence of IFN-γ, as reflected by the sustained values of the dye uptake. The resistance to the IFN-γ-induced cell killing was very similar in all the four tested cultures that expressed the two different antisense RNAs (Figs. 4A, 4B). These data indicate that expression of antisense RNA from groups 1 and 2 protects the HeLa cells exclusively from the IFN- -induced cell death and not from its cytostatic action. It is noteworthy that the antisense RNA expression did not affect the early biochemical steps in the signaling of IFN-γ as deduced from the normal mRNA induction by IFN-γ of the 2-5A synthetase gene in these transfected cells (Fig. 4C). Altogether, it is concluded that among all criteria tested only the death inducing effects of IFN-γ were interrupted by the antisense RNA expression.

IV. Responses of cells transfected with antisense constructs to necrotic cell death

It became interesting at this stage to check whether the antisense RNA expression can also protect the HeLa cells from a necrotic type of cell death. For this, the effect of TNF- added in combination with cycloheximide (CHX) was examined in the various HeLa cell populations. Unlike the effect of IFN-γ, the cell death that was induced by TNF-α + CHX in HeLa cells was very rapid (50% killing after 3 hours) and displayed typical features of necrosis such as swelling of the cells before their lysis. As shown in Table 2,

while the antisense RNA expression from groups 1 and 2 protected the cells from the IFN-γ-induced cell killing, there was no protection from the TNF-α-induced necrotic cell death. All the examined HeLa cell transfectants were killed by the TNF + CHX combination with similar time kinetics and at the same efficiency. Northern blot analysis demonstrated that the levels of the antisense mRNA transcripts in 256-t1 cells were not reduced by the TNF + CHX treatment at 5 hours (not shown) thus excluding the possibility that loss of the antisense RNA expression, caused by the treatment, may be the reason for lack of protective effects from the necrotic cell death. This further suggests a certain specificity of the protective mechanisms regarding the type of cell killing.

Table 2: Expression of antisense RNA (from groups 1 and 2) protects from the IFN-γ-induced programmed cell death but not from the TNF-induced necrotic cell death. (A=540 nm).

		DHFR-t1	DHFR-t2	230-t1	255-t1	256-t1
14 days	No treatment	0.396	0.345	0.385	0.324	0.336
	IFN-γ	0.026	0.017	0.136	0.158	0.159
5 hours	No treatment	N.D.	0.148	0.130	N.D.	0.140
	TNF-α + CHX	N.D.	0.053	0.026	N.D.	0.022
20 hours	No treatment	0.211	0.248	0.223	0.173	0.190
	TNF-α + CHX	0.002	0.001	0.003	0.0015	0.002

Each treatment was done in quadruplicates and the average values of dye uptake, measured by the OD at 1=540 nm, is presented at the indicated time intervals. The SD was between 2-4%. N.D, not done.

V. Cloning of DAP-1 cDNA and determination of amino acid sequence.

An HL-60 cDNA library constructed in λgt10 vector was screened with the cDNA insert of pTKO1-230. Two independent clones, λ1 and λ2, almost completely overlapping and carrying cDNA inserts of about 2.3 Kb were analysed. λ1 cDNA clone encompasses the 5'-untranslated region, short coding region(s) and a relatively long 3'-untranslated region that constitutes more than 60% of the cDNA clone (Fig. 5).

The nucleotide sequence of the cDNA carried by λ1 and its predicted amino acid pattern are presented in Fig. 6. This cDNA is 2232 bp long and contains a potential polyadenylation signal ATTAAA at its 3' end. The open reading frame (ORF) is very short, starting from the initiation codon at nucleotide positions 160-162 and ending at termination codon TGA at positions 466-468. This ORF is preceded by an extremely GC-rich 5'-untranslated region and potentially codes for a protein consisting of 102 amino acids with calculated MW of 11.2 kDa. The amino acid composition predicts a basic protein (isoelectric point = 10), rich in prolines (15%) which displays two blocks of charged residues, one in the middle and the other at the 3' end of the protein. The high proline content may cause some anomalies in the protein's migration on gels. Search for motifs ("Motifs" program; GCG Software Package) indicated that the protein contains two potential sites for case in kinase II phosphorylation at positions 3 and 36, a single potential protein kinase C phosphorylation site at the C-terminus (position 91) and a consensus phosphorylation site of the cdks at position 51. In addition, the protein contains the consensus sequence RGD at position 65-67, a tripeptide that in some proteins plays a role in cell adhesion, and a potential SH3 binding motif, SPSPP, at position 49-53 (Cowburn (1994) Struc. Biol. 1, 489-491). No indications for the presence of signal peptide or transmembranal domain have been found (SAPS prediction; Brendel et al., (1992) PNAS

USA, 89:2002-2006). The amino acid sequence showed no significant homology to known proteins.

Fragment #230 was used as a probe on Southern blots containing human genomic DNA, digested with various restriction enzymes that do not cut it. A single band was visualized upon hybridization with DNA cleaved with EcoRI, BamHI, PstI and XbaI, suggesting the existence of a single copy gene (not shown). This new gene was termed DAP-1 (Death Associated Protein-1).

In vitro translation assays in reticulocyte lysates confirmed that the predicted ORF codes for the major 15kDa protein translated from the cloned 2.4 Kb transcript. The full length cDNA insert as well as four subclones that span different regions of the molecule (i.e., p6, p5, p8, and p4; see Fig. 5) were transcribed and translated in vitro. Among all the tested subclones, only the 5' 1 Kb portion of the DAP-1 cDNA (p6) directed the *in vitro* synthesis of proteins (Fig. 1C). The major translated product migrated on gels as a 15 kDa protein. Mutation at the ATG codon at position 160-162 (ATG to GGC) completely eliminated the synthesis of the 15 kDa protein, thus confirming the position of the start point of this protein (data not shown). In addition to the 15 kDa protein product, a second protein of 22 kDa was also translated at lower efficiency from λ1 and the p6 cDNAs (Fig . 1C). Its translation was not influenced by the elimination of the ATG codon at position 160 but the protein was shortened to a size of 16 and 18 kDa upon cleavage of the p6 subclone with DraI and BstYI restriction endonucleases, respectively (not shown; for restriction map see Fig. 5). These criteria fit another potential open reading frame, which is detected in the nucleotide sequence in a different phase with respect to the first ORF (Fig. 6). It starts at the ATG codon (positions 287-289) and ends at termination codon TGA (positions 816-818). It has the potential to code for a protein consisting of 176 amino acids with a

calculated molecular weight of 19.9 kDa, and has no significant homology to any known proteins.

To analyze the expression of the major DAP-1 protein in cells, rabbit polyclonal antibodies were prepared against the bacterially produced 15kDa protein. The affinity purified antibodies recognized on immunoblots two closely migrating proteins in extracts of HeLa cells; the lower band co-migrated on gels with the bacterially produced 15 kDa DAP-1 protein. The slower migrating form may represent a post-translationally modified version of the protein. In the HeLa cell transfectants, 230-t1 and 255-t1, expressing the elevated levels of antisense RNA that develop in the presence of IFN-γ (15 to 1 ratio), the DAP-1 protein levels were reduced by 75% and 78%, respectively, as compared to the DHFR-tranfected cultures (Fig. 1D). The two upper non specific bands (that are not competed with excess of the bacterially produced DAP-1) were not affected by the antisense expression, thus supporting the selectivity of the effect.

VI. Cloning of DAP-2 and determination of amino acid sequence

As mentioned above, expression studies indicated that the double-stranded cDNA fragment #256 (367 bp in size) hybridized on Northern blots to an endogenous 6.3 Kb mRNA transcript. The same single 6.3Kb mRNA transcript was detected in HeLa (parental and transfectants) and in K562 cells when the full length cDNA (see below) was used as a probe on Northern blots (Fig. 2B). The cDNA insert from pTKO1-256 was therefore used to screen a K562 cDNA library.

Approximately $4x10^6$ pfu were screened with the #256 cDNA insert and 40 positive clones were isolated after two rounds of sequential walking screening. The sequencing was performed on an Applied Bio-systems DNA sequencer 373 A. Sequence uniqueness and relatedness were determined

using FASTA (GCG software package) at the nucleotide level and FASTA, BLASTP, and BLOCKS programs at the amino acid level (S. Henikoff and J. G. Henikoff, Nucleic Acids Res. 19, 6565 (1991).

Two clones, λ29 and λ32, were chosen for sequencing (Fig. 7). The resulting composite sequence of both cDNAs consists of 5886 nucleotides and contains a poly A tail that starts at position 5872 and is preceded by two poly-adenylation signals AATAAA (Fig. 8). The 3'-untranslated region also contains two ATTTA instability motifs found in the 3'-noncoding portions of short-lived mRNAs (G. Shaw and R. Kamen, Cell 46, 659 (1986)). The mRNA contains a single long open reading frame that starts at position 337, ends at position 4605 and potentially codes for a protein of 1423 amino acids (Fig. 8). The calculated molecular weight of the protein product is about 160 kDa. Affinity purified polyclonal antibodies were raised against the N-terminal 20 amino acid peptide of the protein. These antibodies recognized on immunoblots a 160kDa recombinant protein that was produced in COS-1 cells after transfection with a vector that expressed the entire coding region of the cDNA (Fig. 2D). These antibodies reacted in HeLa cells with an endogenous protein of the same size. In the antisense RNA expressing cells, 256-t1 and 256-t2, the steady state levels of the 160kDa protein were 10 and 5 fold lower than in the DHFR control cells while a non relevant protein, vinculin, displayed similar expression levels in all HeLa cell transfectants (Fig. 2D). Thus, expression of anti-sense RNA from pTKO-1 plasmid #256 in HeLa cells resulted in a significant reduction in the amount of the corresponding protein.

We were able to define several known domains and motifs that are present in this protein. Its extreme N-terminus is composed of a protein kinase domain that spans 255 amino acids from position 13-267. On the basis of its structure, it is likely to be a serine/threonine type of protein kinase having a classical composition of XI subdomains with all conserved motifs present

(Fig. 8) (S. K. Hanks and A. M. Quinn, Methods Enzymol. 200, 38 (1991)). This novel kinase was termed DAP-2 or DAP-kinase (Death Associated Protein-kinase).

The kinase domain falls into a family of that of calmodulin-dependent kinases. The homology to known kinase domains that constitute this group, including the myosin light chain kinases, ranges between 34%-49% (Fig. 9A). Three main differences distinguish the kinase domain of DAP-kinase from other members of calmodulin-dependent kinase family: 1) Subdomain II is relatively long and has a stretch of basic amino acids (KKRRTKSSRR); 2) Subdomain III mostly resembles that of the cell cycle dependent kinases (Fig. 9B). Interestingly, the typical sequences of the cell cycle dependent kinases (PSTAIRE, PSSALRE, PCTAIRE, KKIALRE) are located in subdomain III; and 3) Subdomain VII is extremely short and consists of only 7 amino acids.

Right downstream to the kinase domain there is an additional stretch of homology that is present in almost all members of the family of calmodulin-dependent kinases, and was implicated in calmodulin-recognition and binding; B. P. Herring, J. T. Stull, P. J. Gallagher, J. Biol. Chem. 265, 1724 (1990); M. O. Shoemaker *et al.,* J.Cell. Biol. 111, 1107 (1990); F.H. Cruzalegui *et al.,* Proc. Nath. Acad. Sci. USA 89, 12127 (1992)). Down-stream of the calmodulin-recognition domain, an ankyrin repeats domain was identified spanning 265 amino acids from position 365 to 629. It is composed of 8 repeats of 33 amino acids each, not separated by spacers except for a single proline residue that separates three N-terminal repeats from five C-terminal ones (Figs. 8 and 9C). Ankyrin repeats are involved in protein-protein interactions in a variety of proteins (P. Michaely and V. Bennett, Trends in Cell Biology 2, 127 (1992)), but were not described before in the context of serine/threonine kinases. One tyrosine kinase carrying ankyrin repeats has been recently identified in Hydra vulgaris (T.A. Chan *et al.,* Oncogene 9, 1253 (1994)). In the DAP-kinase, the 8 ankyrin repeats may mediate the interaction with a putative effector or a regulatory molecule, or

influence the substrate selectivity and/or stability of the kinase-substrate interactions.

Immediately downstream to ankyrin repeats there are two subsequent potential P-loop motifs, ALTTDGKT and GHSGSGKT, identified through the consensus sequence, G[A]XXXXGKT[S]. Comparison of DAP-kinase potential P-loop motifs to the corresponding consensus sequences within seven ATP or GTP-binding protein families demonstrates that only the 3' P-loop has some similarity to P-loop consensus of elongation factors, ATP synthase b-subunits and thymidine kinase. Actually, a stretch of 33 amino acids following the eighth ankyrin repeat that encompasses the putative 5' P-loop, may represent a ninth ankyrin repeat that is less conserved than others. DAP-kinase also carries multiple potential sites for post-translational modifications, and has neither transmembranal domain nor signal peptide. The Prosite bank search, using the program Motifs (GCG Software Package) revealed that the DAP-kinase protein contains a consensus sequence for the C-terminal amidation site at position 1376 (this suggests that 47 C-terminal amino acids can be cleaved from the protein body). It also contains consensus sequences for six N-glycosylation sites, and potential phosphorylation sites for cAMP-dependent kinase (six), casein kinase II (twenty eight) and protein kinase C (twenty).

Altogether, the deduced amino acid sequence of the DAP-kinase suggests that a very unique type of calmodulin-regulated serine/threonine kinase has been rescued. The combination of serine/threonine kinase domain, ankyrin repeats and additional possible ATP/GTP binding sites outside the kinase domain in one protein (Fig. 10) has not been previously described. A size of 160 kDa is rare among serine/threonine kinases and DAP-kinase is actually the largest calmodulin-dependent kinase known to date. The ability of DAP-kinase to bind calmodulin, recently confirmed in yeast two hybrid system (not shown), is consistent with the notion that in many cases programmed cell death is Ca^{2+} dependent (S. Sen, Biol. Rev. Camb. Philos.

Soc. 67, 287 (1992); S. Lee, S. Christakos, M. B. Small, Curr. Opin. Cell. Biol. 5, 286 (1993)). Moreover, it has been recently reported that calmodulin antagonists inhibited the glucocorticoid-induced apoptosis (D. R. Dowd, D. P. Mac, B. S. Komm, M. R. Haussler, R. Miesfeld, J. Biol. Chem. 266, 18423 (1991)), and that inhibitors of myosin light chain kinases blocked the TNF-induced apoptotic cell death (S.C. Wright, H. Zheng, J. Zhong, F.M.Torti, J.W. Larrick, J. Cell. Biochem. 53, 222 (1993)).

In order to verify that DAP-2 is truely a kinase, COS cells were transiently transfected with an expression vector (PECE-FLAG) that carries a fragment of the λ29 cDNA that encompasses the entire coding region (from the abovementioned start ATG to the first EcoRI site at the 3' end). Cell lysates were immunoprecipitated by anti-FLAG monoclonal antibodies and washed immunoprecipitates were assayed for *in-vitro* autophosphorylation in the presence of calmodulin and Ca^{2+}. As shown in Fig. 2C, a single phosphorylated band of 160 kDa appeared upon fractionation of the *in-vitro* reaction products on polyacrylamide gels. This experiment provides the first direct proof that the recombinant protein has intrinsic kinase activity, as suggested by the predicted amino acid structure.

VII. Assessment of *in vitro* DAP-kinase activity

(A) Experimental Procedures

1. Cell culture

The HeLa human epithelial carcinoma cells, COS-7 monkey kidney cells, SV-80 cells (human fibroblasts transformed with SV0-40 large T-antigen), and REF-52 rat embryo fibroblasts, were grown in DMEM (BioLab) supplemented with 10% FCS (Gibco), 4 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. HeLa-tTA were grown in the presence of 200 μg/ml G-418 (Gossen and Bujard, 1992). Transfections were performed by the standard calcium phosphate technique. Recombinant human

interferon-γ ($3x10^7$ U/ml) was purchased from PeproTech. Nocodazol was purchased from Sigma, Latronculin A was a gift from A.D. Bershadsky of the Weizman Institute, Rehovot, Israel.

2. Plasmid construction

DAP-kinase expression constructs for transient transfections into SV-80, REF-52 or COS cells and for stable transfections into the HeLa cells were prepared in either the pECE (Deng & Karin, 1993) or pCDNA3 (In Vitrogen) vectors. In all the constructs the DAP-kinase sequences were tagged with the FLAG epitope at their N-terminus. In C-terminal deletion constructs the DAP-kinase sequences were fused to the FLAG epitope via the Nde I restriction site that was introduced at the initiation ATG codon by oligonucleotide directed mutagenesis. In other constructs DAP-kinase sequences were fused to the FLAG epitope via the corresponding restriction sites. C-terminal deletion constructs: 1-1271, 1-835, 1-641 and 1-305, - were prepared by digestion of the DAP-kinase cDNA with Hind III (nt 4146), Xba I (nt 2838), Bgl II (nt 2256), and EcoR V (nt 1247), respectively. The full-length DAP-kinase cDNA construct reaches the EcoR I site at position 4932 of the 3′ UTR. DAP-kinase expression constructs 305-641, 641-835, and 641-1423 contain cDNA fragments obtained by double digestion with EcoRV and Bgl II (nt 1247-2256), Bgl II and Xba I (nt 2256-2838), or by digestion with Bgl II (nt 2256-4827), respectively. Three DAP-kinase mutants: K42A, CaM and Cyto, were prepared using oligonucleotide directed mutagenesis with the 5′–GTATCCCGCCGCATTCATCAAGA-3′, 5′–CAGCATCCCTGGATCAAGTCCAGAAGTAACATGAGT-3′, and 5′–AAGACGGCAGAAGATCTAGAAGAGCCCTAT-3′ oligonucleotides, respectively. All the nucleotide numbers are given according to X76104.

DAP-kinase was expressed transiently in HeLa cells from the tetracycline repressible promoter as a bicistronic message with the LacZ sequences. DAP-kinase sequence was tagged with the HA epitope at the

N-terminus via the Nde I site introduced at the initiation ATG codon. The vector for expression of DAP-kinase-LacZ bicistronic message was prepared by insertion of BH-LacZ fusion gene from pUT535 vector (Cayla) into the Not I site of pSBC vector (Korchhoff *et al.*, 1995). The resulting vector was named pSBC-b1.

(B) Results

The deduced amino acid structure of DAP-kinase protein predicts a few functional motifs and domains as depicted in Fig. 31. The amino terminus is composed of a protein kinase domain of the serine/threonine type (Deiss *et al.*, 1995), that spans 255 amino acids from position 13 to 267. In order to measure the kinase activity an *in vitro* immune complex kinase assay was developed for DAP-kinase. FLAG-tagged wild type DAP-kinase, or DAP-kinase mutants, were transiently expressed in COS cells. DAP-kinase proteins were immunoprecipitated by the anti-FLAG antibodies and were subjected to *in vitro* kinase assay, in the presence of 0.5mM Ca^{2+} and 1mM recombinant calmodulin (CaM). Two mutant versions of DAP-kinase were used in this experiment: a C-terminus truncated DAP-kinase that lacks the last 152 amino acids - a region that contains the death domain, and the serine/threonine rich stretch of amino acids (Feinstein *et al.*, 1995) (named DAP-kinase 1-1271 - DD; Fig. 31), and a mutant in which a conserved lysine in the kinase subdomain II (at position 42) was substituted with alanine (DAP-kinase-K42A). The latter mutation, was shown in other kinases to interfere with the phosphotransfer reaction, giving rise to a catalytically inactive protein (Hanks & Quinn, 1991).

As can be seen in Fig. 32, the recombinant DAP-kinase protein that was present in the immune complex was phosphorylated *in vitro* resulting in a prominent ^{32}P - labeled band at the expected protein size. In contrast, the mutant DAP-kinase -K42A failed to be phosphorylated, suggesting that the mutation indeed inactivated the enzyme, and that the label of DAP-kinase

resulted from autophosphorylation. The homology of the kinase domain of DAP-kinase to the myosin light chain kinase (MLCK) (Deiss *et al.,* 1995), prompted the testing of the myosin light chain (MLC) as a potential exogenous substrate for the *in vitro* DAP-kinase assays. As can be seen in Fig. 32, DAP-kinase, but not its catalytically inactive mutant (DAP-kinase-K42A), phosphorylated the MLC under the *in vitro* kinase assay conditions. The truncated DD mutant, DAP-kinase 1-1271, was capable of undergoing autophosphorylation as well as phosphorylating the MLC. This indicates, first, that the region of the C-terminus, especially the most terminal stretch of amino acids that is rich in serines and threonines (Feinstein *et al.,* 1995), is either not subjected to autophosphorylation or most probably is not the sole target for that activity; and second, that the 152 C-terminal amino acids do not participate in recognition of the MLC as a substrate. The amount of the recombinant DAP-kinase protein present in each immune complex was determined by reacting the blots, after the visualization of the ^{32}P signals, with anti-FLAG antibodies (Fig. 32).

VIII. DAP-kinase is a calmodulin-regulated serine/threonine kinase.

(A) Experimental Procedure

1. Calmodulin overlay

Transfection into COS cells, preparation of cell lysates, SDS-PAGE, and transfer of proteins to nitrocellulose, were performed as previously described (Deiss *et al.,* 1995). Protein extracts (300 μg per lane) from COS cells, nontransfected or transfected with FLAG-DAP-kinase or DAP-kinase mutants were run on 7.5% SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was preincubated for 30 minutes in calmodulin binding buffer (50mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM $CaCl_2$) containing 1% non-fat dry milk powder. Recombinant ^{35}S-labeled calmodulin

(Baum *et al.,* 1993) was supplemented, and the membrane was subjected to gentle shaking at room temperature for 16h, washed three times (5 minutes each) in calmodulin binding buffer, dried and exposed to X-ray film. Detection of FLAG-DAP-kinase was done using anti-FLAG antibodies (1:500; IBI, Kodak) and the ECL western blotting detection system as described (Deiss *et al.,* 1995).

2. *In vitro* kinase assay

Cell lysates of COS transfected cells were prepared as described previously (Deiss *et al.,* 1995). Immunoprecipitation of recombinant DAP-kinase protein from 150 μg total extract was done with 20 l anti-FLAG M2 gel (IBI, Kodak) in 200 μl of PLB supplemented with protease and phosphatase inhibitors for 2h at 40°C. Following three washes with PLB, the immunoprecipitates were washed once with reaction buffer (50 mM Hepes pH 7.5, 8 mM $MgCl_2$, 2 mM $MnCl_2$ and 0.1 mg/ ml BSA). The proteins bound to the beads were incubated for 15 min. at 25°C in 50 l of reaction buffer containing 15 Ci [γ-^{32}P] ATP (3 pmole), 50mM ATP, 5 μg MLC (Sigma) and where indicated also 1mM bovine calmodulin (Sigma), 0.5 mM $CaCl_2$, or 3mM EGTA in the absence of $CaCl_2$. Protein sample buffer was added to terminate the reaction, and after boiling the proteins were analyzed on 1% SDS-PAGE. The gel was blotted onto a nitrocellulose membrane and ^{32}P labeled proteins were visualized by autoradiography.

(B) Results

A region, located downstream to the kinase domain (amino acids 280-312; Fig. 32), was predicted to bind CaM, based on sequence homology with the CaM-regulatory domains of several members of the CaM-dependent kinase family (Deiss *et al.,* 1995). A few different assays performed and described below confirmed both the binding of CaM to the DAP-kinase protein and the regulation of the kinase activity by CaM.

The ability of DAP-kinase to bind CaM was first tested by using labeled CaM in an overlay binding assay. In this assay various FLAG-tagged recombinant DAP-kinase constructs were expressed in COS cells and the protein extracts were electrophoresed on SDS-PAGE, blotted to nitrocellulose membranes, and reacted with ^{35}S-Met labeled recombinant CaM (Baum *et al.*, 1993). The wild type DAP-kinase was tested, as well as a deleted version of DAP-kinase that lacks the calmodulin regulatory and binding region (i.e., amino acids 266-312; named DAP-kinase- CaM), and the previously mentioned DD mutant. The same blots were also reacted with anti-FLAG antibodies to confirm the presence of the recombinant protein appearing at the predicted size in each slot. Both, the wild type DAP-kinase and the truncated DD, were capable of binding the labeled CaM, whereas the DAP-kinase- CaM failed to do so (Fig. 33A).

The ability of DAP-kinase to bind CaM was further confirmed by using the yeast two-hybrid selection system (Fields & Sterglanz, TIG 10:286-292, 1994). In this assay the region comprising the end of the kinase domain, the CaM regulatory region, the ankyrin repeats domain, and the first P-loop (see Fig. 31 for details), was used as a bait to fish interacting proteins from the HeLa expression cDNA library (Clontech). About 90 positive clones were obtained, all of them being identical to the human CaM full length cDNA. The rescued CaM clones also reacted in the yeast system with a truncated construct of DAP-kinase which was exclusively comprised of the end of the kinase domain and the CaM regulatory domain (amino acids 251-364). Altogether, the CaM overlay assays and the interactions between DAP-kinase fragments and calmodulin in the yeast two hybrid system, confirmed the prediction that DAP-kinase binds CaM through the conserved domain that lies downstream to the kinase domain.

The Ca^{2+} / CaM regulation of the kinase activity was further investigated in the *in vitro* kinase assays. In the absence of Ca^{2+} / CaM, both the autophosphorylation and the MLC phosphorylation by DAP-kinase were 8-10

fold lower than the phosphorylation in the presence of Ca^{2+} / CaM (Fig. 33B). Interestingly, the CaM regulatory domain deletion mutant (DAP-kinase- CaM) displayed a high level of enzymatic activity in the absence of Ca^{2+} / CaM, suggesting a negative regulatory function of this region that could be relieved by the interactions with calmodulin (Fig. 33C). These results were consistent with the stimulatory effects which were imposed by the deletion of this region in other CaM-dependent kinases (Shoemaker *et al.* , 1990). We therefore concluded from the *in vitro* kinase assays that the kinase activity of DAP-kinase is regulated by Ca^{2+} / CaM, and that the removal of the CaM regulatory domain generates a deregulated kinase that is constitutively active.

This type of mutation is an example of a 'gain of function' mutation, i.e. a mutation which results in the DAP gene product having an increased cell death-promoting activity. Such a mutation would be useful, e.g. in the death promoting aspect of the invention.

IX. Ectopic expression of DAP-kinase induces the death of HeLa cells

(A) Experimental Procedure

1. Detergent extraction assay

Sub-confluent cultures of COS transfected or HeLa cells, grown on 9cm plate, were washed once with PBS and then with MES buffer (50mM MES pH 6.8, 2.5 mM EGTA, 2.5 mM $MgCl_2$) where indicated, HeLa cells were pretreated with 1mg/ml nocodazol for 0.5 hour, or with 5mM latrunculin A for 1 hour, before extraction. The cells were extracted for 3 min. with 0.5 ml of 0.5% Triton X-100 in MES buffer supplemented with protease inhibitors. The supernatant (the soluble fraction- Sol) was collected, centrifuged for 2 min. at 16,000x g at 4°C, and the clear supernatant was then transferred to new tubes. Two volumes of cold ethanol were added and the tubes were incubated at -20°C for overnight, centrifuged 10 min. at 16,000x g

at 4°C and resuspended in 200 l of 2x protein sample buffer without dye. The detergent insoluble matrix (InSol) remaining on the plate was extracted in 200 µl of 2x protein sample buffer, scraped from the plate with rubber policeman and collected into tube. The samples were loaded on 10% SDS-PAGE, 100 µg protein extracts were loaded on each lane from the Sol fraction, equivalent volumes of InSol were loaded. Analysis of the proteins was done using anti FLAG antibodies (Kodak), the monoclonal antibodies against DAP-kinase (1:1000 dilution; Sigma), anti-tubulin antibodies (1:2000 dilution; Sigma) or polyclonal anti actin antibodies (1: 100 dilution; Sigma) as described above.

2. Immunostaining of cells

Transfected cells (SV-80, REF-52 or COS cells) were plated on glass cover-slips (13mm diam.), 20,000 cells/well in 1 ml medium within a 24-wells plate. After 48 hours, the cells were washed twice with PBS, fixed and permeabilized simultaneously. This was carried out by incubating the coverslips for 5 minutes in a mixture of 3% paraformaldehyde and 0.3% Triton X-100 in PBS, and then incubating with 3% paraformaldehyde alone for additional 20 minutes. The cells were washed three times in PBS and then incubated in blocking solution (5% normal goat serum and 1% BSA in PBS) for 60 minutes. The cells were incubated with 30ml of the first antibody (anti FLAG 1:300) for 60 minutes at room temperature, then washed three times in PBS and incubated for 30 minutes with 30ml of rhodamine-conjugated goat anti mouse antibodies (dilution 1:200; Jakson Immuno Research Lab.) DAPI (0.5 µg/ ml; Sigma) and fluoresceine-conjugated phalloidin (1:100; Molecular Probes Inc.) were added at this step. The coverslips were washed three times in PBS, drained and mounted in Mowiol. Microscopy was done under conditions of fluorescent light. Photography was done using Kodak TMX400 film.

3. X-Gal staining

To detect LacZ expression, cells were fixed with 3% paraformaldehyde for 5 min., rinsed twice with PBS and stained for 3 h in X-Gal buffer containing 77mM Na_2HPO_4, 23mM NaH_2PO_4, 1.3mM $MgCl_2$ 1mg/ ml X-Gal, 3mM $K_3Fe(CN)_6$ 3mM $K_4Fe(CN)_6$. Reaction was stopped by 70% ethanol. Photography was done under phase microscopy using Kodak Ektachrome 160T.

(B) Results

The first indication that attributed a function to DAP-kinase, as a positive mediator of cell death, was based on the finding that its reduced expression by the anti-sense RNA protected HeLa cells from apoptotic cell death initiated by the IFN-γ receptors. It was therefore interesting to test whether elevated levels of DAP-kinase protein, generated by the ectopic expression of the full length sense cDNA, may cause cell death on its own without any external stimulus.

In order to express DAP-kinase in mammalian cells, the full length cDNA was cloned into pcDNA3 vector (InVitrogen), under the control of the CMV promoter. Similar constructs were prepared containing the catalytically inactive DAP-kinase-K42A mutant, and the CaM regulatory domain deletion mutant (DAP-kinase CaM). DAP-kinase constructs, as well as the empty vector were transfected into HeLa cells by the calcium phosphate co-precipitation technique. After 2-3 weeks of growth in selection medium (G-418), the drug resistant cells were stained with crystal-violet. It was found that transfection with the wild type DAP-kinase significantly reduced the number of surviving colonies compared to the transfections with the empty vector (Fig. 34A). The inhibitory effect was even more pronounced upon transfections with the constitutively active DAP-kinase- CaM mutant, suggesting that this mutant had the most prominent growth restrictive effects. In contrast, the catalytically inactive DAP-kinase mutant did not reduce at all the number of colonies. Instead, the number of colonies generated by

transfection with the K42A mutant was slightly increased, compared to the transfections with the empty vector, and the size of individual colonies was often larger (Fig. 34A). Transfection with the catalytically inactive DAP-kinase mutant therefore seemed to confer some growth advantage to cells during the process of colony formation. These results were repeated in six independent experiments, with different preparations of plasmid DNA. They were also repeated with other types of expression vectors (not shown). These data provided the first indication that the ectopic expression of DAP-kinase was not compatible with continuous cell growth, and that this feature depended on the intrinsic kinase activity. They also provided the first hint that the catalytically inactive mutant of DAP-kinase may have a dominant-negative function, an issue that was examined later under the restrictive effects of IFN-γ (see below).

In order to determine more precisely the fate of the cells and to understand the basis for the suppression of colony formation, the cells were examined two days after the transfection with the DAP-kinase gene. In these experiments, the LacZ marker gene was used to facilitate the recognition of the transfected cells that ectopically express the DAP-kinase. A vector was constructed for this purpose containing the internal ribosomal entry site (IRES) of poliovirus and thus directing the expression of both LacZ and the wild-type DAP-kinase genes within a single bicistronic message. The bicistronic mRNA was expressed from the tetracycline-repressible promoter (Gossen & Bujard, PNAS 89:5547-5551, 1992). The morphology of LacZ containing blue cells was determined 48 hours post transfection, in cultures which were maintained in the absence of tetracycline to allow the continuous expression of both genes. It was found that 34% of the lacZ containing cells which expressed the wild-type DAP-kinase displayed the morphology of apoptotic cells, i.e., cell shrinkage and rounding up followed by detachment from the plates. In contrast, in the control vector a background of less than 5%

apoptotic cells was detected (Fig. 34B). Altogether, the morphological assessments and the colony formation assays suggest that overexpression of DAP-kinase promotes cell death, thus reinforcing the role of DAP-kinase as a positive mediator of cell death.

X. The catalytically inactive DAP-kinase protects cells from the IFN-γ-induced cell death

The hypothesis that DAP-kinase-K42A mutant may function in a trans-dominant negative manner was tested. This was done by checking whether the catalytically inactive mutant kinase may protect HeLa cells from the IFN-γ induced cell death, similar to the protection conveyed by the anti-sense RNA expression. In this experiment the empty pcDNA3 vector and the one containing the DAP-kinase-K42A mutant, were transfected into HeLa cells. Forty eight hours after transfection, the cells were split and subjected to double selection with 700 μg/ml G-418 and 200 U / ml of IFN-γ. Under these stringent conditions, the transfectants that expressed the control vector were efficiently killed, and the background of G-418 resistant cells was extremely low. In contrast, transfection with the K42A mutant had significantly increased the number of surviving cells (Fig. 35A). On average, the relative number of colonies that survived in the presence of IFN-γ was 5-fold higher in the K42A transfectants than in the corresponding pcDNA3 transfectants (Fig. 35B). Also the average cell number per colony was higher in the K42A transfectants. These results indicate that the K42A mutant can protect HeLa cells from the IFN-γ induced cell death, presumably by acting in a dominant negative manner, thus interfering with the normal function of the endogenous DAP-kinase.

This is an example of a mutation which can result in the neutralization of the endogenous DAP gene product. Such a mutation would be useful, e.g. in the death preventing aspect of the invention.

XI. Cytoskeleton localization of DAP-kinase

One of the key questions in understanding the DAP-kinase mode of action concerns its intracellular localization. In order to define, by immunofluorescent staining, the intracellular localization of DAP-kinase, we have transiently transfected SV-80 human fibroblasts with the aforementioned FLAG-DAP-kinase-K42A construct, and immunostained the cells with anti-FLAG antibodies. The K42A mutant was chosen to avoid death-related morphological changes upon overexpression (transfection of SV80 cells with wild-type DAP-kinase induced cell death similar to that observed in HeLa cells, as detailed below).

The FLAG-DAP-kinase-K42A was stained as a network of delicate fibers reaching the cell periphery; nuclei were not stained (Fig. 45A). The same pattern was also revealed by staining with anti-DAP-kinase monoclonal antibodies (not shown). This was the first indication which suggested a cytoskeletal localization of DAP-kinase protein. Double staining of the transfectants with anti-FLAG antibodies and with fluoresceine-conjugated phalloidin which binds to actin fibers, revealed a considerable overlap (Fig. 45A). In contrast, there was no overlap with the microtubule staining (not shown).

The cytoskeletal localization of DAP-kinase was subsequently confirmed by the biochemical fractionations of both the endogenously and exogenously expressed protein. We used the well elaborated protocol of gentle cell extraction with nonionic detergent (0.5% Triton X-100) that removes lipids and soluble proteins, leaving intact the detergent insoluble matrix composed of the nucleus, the cytoskeleton framework, and cytoskeleton-associated proteins. In non-transfected HeLa cells, the endogenous DAP-kinase (recognized by monoclonal antibodies raised against the C-terminus of the protein) appeared exclusively in the detergent insoluble

fraction (Fig. 45B). In contrast, β-tubulin and actin that both have a constant soluble pool, were found both in the detergent soluble and insoluble fractions. We used nocodazol, a microtubule disrupting drug, to change the solubility of -tubulin. As can be seen in Fig. 45B, after treatment of HeLa cells with nocodazol, all of the β-tubulin protein was found in the soluble fraction, whereas the solubility of both DAP-kinase and actin did not change. On the other hand, after treatment of the cells with latrunculin A, a microfilament disrupting agent, a substantial portion of DAP-kinase was found in the soluble fraction. Here, actin was found almost exclusively in the soluble fraction, whereas the solubility of β-tubulin did not change. These results in combination with the double immunostaining suggest that DAP-kinase might be localized to the microfilament system of the cytoskeleton.

The detergent extraction assay was further used to map the region within the DAP-kinase that associates with the cytoskeleton. For this purpose, we used COS cells transfected with FLAG-DAP-kinase, in which the pattern of staining with anti-FLAG antibodies was similar to that observed in the aforestudied SV-80 and HeLa cells (Fig. 46A). A series of constructed DAP-kinase deletion mutants in the pECE-FLAG or pcDNA3-FLAG expression vectors were transfected into COS cells. These transfected COS cells were subjected to detergent extraction as described above, and the immunoblots were reacted with the anti-FLAG antibodies to monitor in each case the elution profile of each DAP-kinase mutant product. The results are summarizes in Fig. 46B. From the detailed analysis it was concluded that the region comprising amino acids 641-835 contributes to the detergent insolubility of DAP-kinase and therefore it is a critical region responsible for the association with the cytoskeleton. Its deletion interfered with the cytoskeletal association, and conversely, this region by itself was detergent insoluble. Interestingly, fragments containing the ankyrin repeats without the cytoskeletal binding domain were completely detergent soluble.

The intracellular localization of DAP-kinase may be relevant to the cytoskeletal alterations that occur during the IFN-γ-induced death of HeLa cells. Staining of actin with the phalloidin showed that after the treatment with the cytokine a complete distruption of microfilament organization took place, and stress fibers disappeared (Fig. 47A). The loss of stress fibers occurred before the typical nuclear alterations, which consist of chromatin condensation and segmentation (Deiss et al., 1995), had taken place. In order to follow the possible effects of DAP-kinase overexpression on the cytoskeleton network, REF-52 fibroblasts possessing a well organized actin cytoskeleton were used.

The constitutively active FLAG-DAP-kinase- CaM mutant was transiently transfected into these cells. After 48 hours, the cells which were positively stained with the anti FLAG antibodies, were examined for nuclear and cytoskeletal alterations, in comparison to the adjacent non-transfected cells. This was achieved by triple staining with DAPI (for nuclei) and phalloidin (for the microfilament system) (Fig 47B). It was found that the FLAG positive cells displayed a disrupted pattern of microfilament staining that was reminiscent of the cytoskeletal alterations occurring in the IFN- -treated cells. No signs of chromatin condensation or fragmentation could be detected at this time point in the DAP-kinase-transfected cells (Fig. 47B). In contrast, transfections with the truncated catalytically active DAP-kinase (DAP-kinase-DEcoRV; amino acids 1-305 in Fig. 46B) which was mislocalized in the cells, and showed a nuclear rather than cytoskeletal staining, did not lead to any cytoskeletal alterations. In these transfections, the FLAG positive cells displayed a normal pattern of microfilament staining which could not be distinguished from the adjacent non-transfected cells (Fig. 47B). These results were repeated in SV80 cells, in which more than 80% of transfectants expressing the FLAG-DAP-kinase-DCaM mutant showed abnormal pattern of microfilament staining, whereas no change was caused by

the DAP-kinase-DEcoRV transfections (not shown). These results suggest a link between the correctly localized active DAP-kinase and the cell death--related cytoskeletal and morphological changes that develop in response to IFN-γ.

Thus, the cytoskeleton localization of DAP-kinase, and perhaps other DAP proteins, can be important with respect to the death-promoting and death-preventing aspects of the invention, e.g. with respect to drugs which can prevent protein localization in the cytoskeleton.

XII. Expression of DAP-1 and DAP-2 proteins in various cells and tissues

Examination of a variety of cell lines and tissues revealed that these two genes are likely to be ubiquitously expressed. Fig. 10 shows the Northern blot analysis of RNA from different hematopoietic cells probed with the DAP-1 cDNA. The 2.4 Kb mRNA transcript of this gene was detected in granulocytes (HL-60) B lymphoid (Daudi) and macrophage (U937) cells. The expression levels in the hematopoietic cells was lower than in HeLa cells. Fig. 11 shows results of examination of the mRNA expression in human embryonic tissues: brain, spleen (predominantly B cells) and liver (predominantly erythrocytes). Again the single 2.4 Kb mRNA transcript was detected in these tissues by the DAP-1 cDNA probe.

The DAP-2 cDNA probe 2 recognized the 6.3 Kb mRNA encoded by this gene in these different tissues (Fig. 11). The embryonal liver and spleen tissues from Down syndrome seemed in this blot to express higher levels of the DAP-2 gene (compared to the GAPDH levels) while the brain tissue from Down syndrome contained higher levels of DAP-1 mRNA than the corresponding normal brain.

XIII. Screening cell lines for DAP-kinase expression

(A) Experimental Procedure

1. Maintenance of cell lines and treatment with 5-Azadeoxy-cytidine- All haematopoietic cell lines (see ATCC for description of various lines) were grown in RPMI 1640 supplemented with 10% complement-inactivated FCS (Gibco-BRL), 100 IU / ml penicillin and streptomycin, and 2mM L-glutamine, at 37°C and 5% $C0_2$. For the bladder carcinoma derived cell lines (see ATCC for description of various lines), DMEM was used. The bladder carcinoma cell lines were plated at $1E^5$ cells /100mm dish, and treated 24 hours later with 5-Aza-2'-deoxycytidine (Sigma Chemical Co., St. Louis, MO.) at final concentration of 1 μM. The medium was changed 24 hours after addition of the drug and every 3 days thereafter. Proteins were extracted 9 and 16 days after treatment for the early passage and late passage, respectively.

2. Northern blot analysis - Total RNA was extracted from the various cell lines using Trireagent (MRC). Samples of 3 μg $polyA^+$ RNA, prepared with oligo-dT Dynabeads (Dynal) as described by manufacturer, were separated on 1% agarose gels, and hybridized to Hybond-N nylon membranes (Amersham), as described (Sambrook,1989). DNA probes were prepared using [$-^{32}$P]dCTP with commercially available random priming kits (Boehringer Mannheim). Prehybridization, hybridization and washing of filters were performed as described (Sambrook,1989).

3. Immunoblot analysis - Cells were harvested and protein extracts were prepared as previously described (Deiss et al.,1995). The protein extracts (200 μg/lane) were fractionated on 7.5% SDS-PAGE. The proteins were transferred to a nitrocellulose filters (Schleicher and Schuell) with a semi-dry semi-phor blotter (Hoefer Scientific Instruments). The mouse anti-human DAP-kinase monoclonal antibodies were from BioMaker (Rehovot, Israel). Anti-human vinculin antibodies were from Sigma. Anti-human DAP3 were

prepared as previously described (Kissil et al., 1995). Immunodetection was carried out using the ECL detection system (Amersham).

(B) Results

When analyzing the expression of the DAP-kinase mRNA transcript in various cell lines, it was found that it was not expressed in a substantial proportion of cell lines derived from human B-cell neoplasms. Nine different cell lines, representing different stages of B cell maturation, were examined. Seven of them- SKW, 697, Daudi, RS4:11, MV4:11, SK-DHL and B380 failed to express detectable levels of the DAPk mRNA. Two cell lines: ALL-1 representing a premature B cell stage (Erikson et al. PNAS 83:1807-11, 1986) and the B-1 representing an early progenitor (Erikson et al.,1986; Cohen et al.,1991) expressed DAP-kinase (Fig. 36 and Table 3). In EBV-immortalized B-cell lines established from normal peripheral B cells, GM1500 and GM607, the DAP-kinase mRNA was present (Fig. *[1]37A). The signals in the DAP-kinase negative cell lines remained below detection limits also when higher amounts of poly A^+ RNA (15 μg) were analyzed (not shown).

Cell line origin	DAP-K expression Positive	DAP-K expression Negative	DAP-K expression <1% (a)
Normal B-cells (2)	(2) GM607, GM1500	(-)	(-)
Bladder Neoplasms (9)	(2) ALL-1, B-1	(7) Daudi, 697, RS4:11, B380, MV4:11, SKW, SK-DHL	(-)
Bladder Carcinomas (14)	(8) RT4, RT112, JO'N, 5637, HT1-197, 253J, J82 SW1710	(4) SCa-BER, T24, 609CR, HT1376	(2) UM-UC-3, VM-CUB-2

Table 1. Summary of cell types that were examined for expression of DAP-kinase at the protein level. The data was grouped according to the origin of the cell line, and the expression pattern of DAP-kinase.

(a) Determined by comparison of signal intensity on western blot to signal levels of the DAP-kinase positive bladder carcinoma cell lines.

Interestingly, the c-Abl mRNA transcripts were expressed in all the examined cell lines. The c-Abl gene is the closest known marker to DAP-kinase (Feinstein et al., 1995) The expression of c-Abl appeared to be normal and the two expected mRNA transcripts were observed in all the cell lines irrespective of whether they expressed DAP-kinase mRNA (Fig. 36B). The undisturbed pattern of c-Abl expression minimized the possibility that lack of DAPk expression is a consequence of gross rearrangements or deletions at 9q34.

The protein analysis confirmed the RNA data. Protein extracts prepared from the various cell lines were examined for the presence of the DAP-kinase protein by immunoblot analysis. It was found that in all the cell lines that did express DAP-kinase mRNA transcript, the protein product with the expected size of 160 kDa, was also detected. In contrast, in cell lines in which DAP-kinase transcript was not detected there was no trace of the DAP-kinase protein (Fig. 36C). The same immunoblots were also reacted with anti-vinculin antibodies as an internal control (Fig. 36D). Together, the RNA and protein data indicate that the absence of DAP-kinase expression is most probably a genuine phenomenon and not an artifact of the assays.

The expression of DAP-kinase protein was then examined in various cell lines of bladder carcinoma origin. This was done by Western blot analysis of protein extracts prepared from 14 different cell lines. Out of the 14 lines examined eight lines expressed DAP-kinase, four showed no detectable DAP-kinase protein expression, and two other lines expressed it at levels lower than 1% in comparison to the DAP-kinase positive lines (Table 3).

These results indicated that loss, or very low levels of DAP-kinase expression also occurs at a statistically significant frequency in bladder carcinoma cell lines. Two DAP-kinase negative bladder carcinoma cell lines were chosen (T24 and HT1376) and treated with 5-azadeoxycytidine in order to test whether the absence of DAP-kinase expression was due to DNA hypermethylation. Treatment of cells with 5-azadeoxycytidine causes the removal of methyl groups from CpG dinucleotides and thus may reverse promoter shut-off/silencing due to hypermethylation and may restore expression of the relevant gene (Jones, 1985). Cells were treated with the drug for 24 hours, washed, and protein extracts were prepared at early and late passages thereafter. The expression of DAP-kinase was analyzed by reacting the immunoblots with anti-DAP-kinase antibodies. It was found that while the DAP-kinase was undetectable before treatment (Fig. 37, lane 1 and 4), the addition of 5-azadeoxycytidine to the growth medium restored DAP-kinase expression and strong signals at the expected protein size were detected early after the drug treatment (Fig. 37, lane 2 and 5). The restored levels of DAP-kinase expression are similar to the average expression levels of DAP-kinase in several DAP-kinase positive bladder carcinoma cell lines that were tested (data not shown). The effect was specific since the expression of two other proteins, which unlike DAP-kinase were initially present in this cell line: vinculin, and DAP-3, was not influenced at all by the 5-azadeoxycytidine treatment (Fig. 37, lanes 1 through 5). Late after drug treatment of the T24 cells (after six passages), the expression of DAP-kinase was again completely abolished, probably due to de novo methylation of the gene (Fig. 37, lane 3).

XIV. Cloning and sequencing of DAP-3, DAP-4 and DAP-5

Clone 259 (DAP-3) was sequenced and used to screen a K562 gt10 cDNA library as described above for DAP-1 and DAP-2. The sequence of the full length cDNA of DAP-3 and the deduced amino acid sequence is shown in Fig. 12.

Clone 253 (DAP-4) was partially sequenced as described above for DAP-1 and DAP-2 and the results are shown in Fig. 13.

Clone 260 was among the rescued vectors described in Table 1 which protected the HeLa cells from IFN-γ-induced programmed cell death. It was isolated as described in the detailed description of the invention (section I(A)). It carried a cDNA fragment of 763 bp and the sequence analysis indicated that it corresponded to a novel gene (named DAP-5). Northern blot analysis indicated that DAP-5 is transcribed into a 3.8 Kb mRNA. DAP-5 mRNA was found to be widely expressed in a variety of normal tissues.

The 763 cDNA fragment was used for screening a cDNA library originating from K562 cells. The phage clone that carried the longest cDNA insert (3.8 Kb) was sequenced. This cDNA clone comprises of an open reading frame (ORF) that corresponds to 940 amino acids, as shown in Fig. 15. The deduced amino acid sequence predicts that the protein is highly homologous, yet not identical, to the translation initiation factor 4 (eIF4 ,p220). Thus, DAP-5 may be regarded as a novel member of what appears to be a family of the eIF4 type of translation initiation factors. Most interestingly, and very much unexpectedly, the 763 bp fragment that was presented in the original clone #260 was inserted in the vector in the sense orientation. In this region (marked by a solid line in Fig. 15; nucleotides 1764-2528) there is an ATG codon that could drive the synthesis of a mini protein that is 230 amino acids long. Indeed, *in vitro* transcription and translation of this fragment yielded a protein of that predicted size, and mutation of this ATG eliminated the mini-protein synthesis. Transfections of HeLa cells with vectors that express the 763 cDNA fragment from the tetracycline regulated promoter protected the cells from cytokine-induced cell death. One possibility is that the mini-protein functions as a dominant negative mutant that competes with the death-inducing properties of the full length protein. Other possibilities also exist.

XV. Expression of the DAP-5 cDNA fragment (#260) exerts a dual effect on HeLa cells.

(A) Experimental Procedure

1. Transfections and selection procedures

Two secondary polyclonal HeLa cell populations, expressing the DAP-5 763bp cDNA fragment from the pTKO1 vector were generated. This was performed by the transfection of subconfluent monolayers of 1.5 $x10^6$ HeLa cells with 40 g of the corresponding plasmid (named pTKO1-260). In parallel, HeLa cells were transfected with a control vector, pTKO1-DHFR (Deiss & Kimchi, 1991). Pools of 10^4 independent stable clones were generated from each transfection. The stable transfectants were maintained in the presence of 200 μg/ml hygromycin B (Calbiochem). Subconfluent monolayers of 1.5 $x10^6$ HeLa-tTA cells were transfected with 15 μg of pSBc-bl plasmid or pSBc-bl plasmid carrying either the #260 fragment (pSBc-bl-260) or its mutant derivatives (single & triple ATG mutants) and selected in the presence of either 10 or 50 μg/ml bleomycin. Pools of 10^2-10^3 independent stable clones were generated from each transfection.

2. *In vitro* translation of DAP-5 protein in reticulocyte lysate

The full length cDNA insert, or the #260 variants, cloned into the Bluescript vector (Stratagene), were used as templates for in vitro transcription from the T7-promoter. These RNAs were then translated in reticulocyte lysates (Promega) using the conventional procedures with [^{35}S]-methionine (Amersham) as a labeled precursor. The reaction products were resolved by fractionation on 12.5% SDS polyacrylamide gel, followed by salicylic acid amplification of the radioactive signal performed as described in Kissil, J. L., et al, J. Biol. Chem. 270:27932-936 (1995). ATG codons at position 1785-1787, 2010-2012 or 2040-2042 were mutated by oligonucleotide

directed mutagenesis (ATG was converted into AAG or TTC or ATC respectively).

3. Preparation of antibodies and immunoblot analysis

The DAP-5 sequence corresponding to amino acids 522-776 of the coding region encompassed in the #260 fragment, was fused in-frame to pGEX1 (GST260). Expression of the glutathione S-transferase (GST) fused chimera was induced in *E. Coli* strain XL1-Blue by IPTG. The GST fused product purified on glutathione beads was used to immunize two rabbits. The antiserum was depleted of the anti-GST antibodies by passing it through CNBr-activated sepharose beads (Pharmacia Biotech Inc.) coupled to GST. Affinity purification was then carried out on CNBr-activated sepharose beads coupled to GST260. In several experiments the signal of the mini-protein in the HeLa cell transfectants was below detection limits.

4. Western analysis

HeLa-tTA cells were harvested and lysed by boiling in Sample buffer. The protein samples were fractionated by 10% SDS-PAGE and then transferred to nitrocellulose filter (Schleicher & Schuell). The blots were reacted with the affinity purified polyclonal antibodies (1:20 dilution) and immunodetection was done using the ECL detection system (Amersham Corp.)

(B) Results

The pTKO1 construct containing DAP-5 cDNA fragment #260, was transfected (in duplicates) into HeLa cells to generate two stable polyclonal cell populations (named 260-t1 and 260-t2). A control polyclonal HeLa cell population (designated DHFR-t1) was obtained by transfection of the pTKO1 vector carrying the DHFR gene. These three transfected cell cultures were subjected to the long-term treatment with IFN-γ. As shown in Fig. 38B, there was a 100 to 200-fold increase in the number of growing colonies in the 260-t1 and 260-t2 cell cultures, as compared to the DHFR-t1 cell population. This means that the total number of colonies which were rescued from the inhibitory effects of IFN-γ by this cDNA fragment, corresponded only to 0.1-1% of the initial cell population. This pattern, in which only a small fraction of cells were protected from programmed cell death was very similar to the effect that was conferred by the anti-thioredoxin RNA (Fig. 4 in Deiss & Kimchi, 1991) and by fragment #253 (not shown), all classified in subgroup II.

The size of exogenous DAP-5 RNA in the 260-t1 and 260-t2 transfectants was 1.7Kb (consisting of 763 bases of the cDNA insert, 800 bases of sequences derived from the expression cassette (Deiss & Kimchi, 1991) and of the poly (A) tail). The expression levels of the exogenous RNA were much lower than those of the endogenous 3.8 kb transcript (Fig. 38A). This stood in sharp contrast to the eight plasmids of subgroup I, whose RNA

products accumulated in HeLa cells in large excess over the endogenous mRNA transcripts (i.e., antisense corresponding to DAP-1, DAP-kinase, DAP-3 and cathepsin D). A more detailed comparison between the two subgroups was performed by hybridization of Northern blots, containing RNA from the different HeLa transfectants, with a common DNA probe derived from the pTKO1 vector. As shown in Fig. 38C, under conditions where the RNA expressed from pTKO1-230 vector (containing the antisense fragment of DAP-1) gave a strong signal, the RNA transcribed from fragment #260 was still below detection limits. Similar low levels of RNA were expressed from other two subgroup II cDNA fragments carried by the same vector, thioredoxin (Deiss & Kimchi, 1991) and DAP-4 (#253) (Fig. 38C). Thus, the low levels of the ectopically expressed RNA in the established polyclonal populations provided a second characteristic feature of subgroup II cDNA fragments. This could reflect either RNA instability, or alternatively the selection of transfectants with low copy number of episomes. The latter seemed to be true since a transcript common to all the transfectants - the mRNA expressed from the hygromycin B resistance gene placed within the pTKO1 under the control of the thymidine kinase promoter - paralleled the expression levels of the inserted cDNA fragments (Fig. 38D). Thus, it was postulated that during the establishment of the polyclonal cell populations, done in the presence of hygromycin B only, cells containing a low copy number of the pTKO1-260 episomes gained significant growth advantage.

To further pursue this possibility, the question of whether indeed high expression levels of the DAP-5 partial cDNA were incompatible with continuous cell growth was tested. For this purpose, a polycistronic vector was constructed (pSBc-bl-260) which directed the expression of a bicistronic message containing both the #260 cDNA fragment and the SH-LacZ, which directs the synthesis of a fused protein conferring resistance to bleomycin and producing b-galactosidase (Cayla). The LacZ was used as a marker to

evaluate the bicistronic mRNA levels in individual cells. The #260 cDNA fragment was translated in a cap-dependent manner, whereas the SH-LacZ fused gene was placed downstream to the poliovirus internal ribosomal entry site (IRES). Since IRES-directed translation is less efficient than the cap-dependent one, high levels of bicistronic message, had to be expressed in order to allow the survival of cells under bleomycin selection. This enforced the system to produce high levels of #260-derived expression products in the cells.

It was found that transfections of HeLa-tTA cells with the pSBc-bl-260 vector, did not yield stable clones in the presence of 50 μg/ml bleomycin, while transfection with the control vector lacking the insert (pSBc-bl) readily generated clones. At lower drug concentrations (10 μg/ml bleomycin), small clones did arise after transfections with the pSBc-bl-260 vector; yet, they slowly died thereafter. This indicated that high levels of expression from #260 cDNA fragment was lethal to cells, and that the #260-dependent cell death displayed slow pattern of killing. The expression levels of the bicistronic mRNA and hence of fragment #260, that were permissive for cell growth, were not sufficient for conferring bleomycin resistance and therefore the drug had to be removed in order to enable further analysis of these transfectants.

The β-galactosidase activity, served to quantify the expression on a single-cell basis in surviving cells. In the pSBc-bl-260-transfected cultures, the extent of blue staining was very weak in all the cells on the plate. In contrast, the polyclonal cell populations obtained from transfection with the control vector, and selected under identical conditions, showed a strong pattern of β-galactosidase staining, exceeding by many fold the one in pSBc-bl-260 transfectants (Fig. 39A). Together, the reduced cloning efficiency and the weak β-galactosidase activity in the survived cells, proved that there was a negative selection against high expression of the #260 cDNA

fragment. We concluded that expression from the rescued DAP-5 cDNA fragment had a dual effect: at low levels it conferred IFN-γ resistance (a property that led to its functional cloning); at higher levels, it was toxic and not permissive to continuous cell growth.

XVI. Fragment #260 directs the expression of a functional mini protein

Three AUG codons, that could serve as potential initiators of translation, were found in the ORF of fragment #260. One was located at the beginning of the fragment and could potentially initiate the synthesis of a 28kDa protein; the two others were located 85 and 75 aa downstream and could give rise to 20 and 18.8 kDa proteins, respectively. *In vitro* translation of the RNA transcribed from the 763 bp DAP-5 cDNA fragment, generated a doublet of proteins that had an approximate size of 28 kDa (Fig. 40A, lane 3). A missense mutation in the first ATG codon completely eliminated the synthesis of these two closely migrating proteins, without affecting the two downstream ones. Missense mutation of the next two ATG codons without affecting the first one, did not interfere with the translation of the doublet; a triple mutation in all potential initiation codons completely prevented the protein translation as did the single mutation in the first ATG codon (Fig. 40A lanes 4-6, respectively). Thus it was concluded that the rescued cDNA fragment could drive the expression of a mini-protein starting at Met 529. This mini-protein was also detected in the aforementioned population of HeLa cells that had been transfected with the pSBc-bl vector harboring the #260 fragment. As shown in Fig. 40B, affinity purified polyclonal antibodies, raised against the recombinant mini-protein, identified two closely migrating proteins with approximate size of 28kDa exclusively in the cells transfected with the pSBc-bl-260 and not in cells transfected with the empty vector.

The question was raised whether the biological effects conferred on the transfected cells by fragment #260 resulted from expression of the mini-protein. For that purpose we have subcloned each of the two mutant cDNA fragments, which failed to be translated in vitro, into the pSBc-bl vector. In contrast to the transfections with the protein expressing DAP-5 fragment, polyclonal cell populations obtained with the mutant ones were established in the presence of bleomycin, with an efficiency similar to that of the control vector. In addition, the β-galactosidase activity in these transfectants was as high as in the cells transfected with the control vector (Fig. 39B). Thus, high levels of expression of mutant DAP-5 cDNA fragment proved to be compatible with continuous cell growth. The translated mini-protein is therefore responsible for the cellular effects that the rescued DAP-5 fragment exerts on cells.

XVII. Characterization of cathepsin D as a DAP molecule

The initial microscopic observations, performed on the different HeLa cells that had been transfected with the individual rescued pTKO1 clones (described in Table 1), indicated that plasmid pKTO1-229 (group 6) conveyed similar effects to those conferred by the plasmids from group 1. It reduced the susceptibility of the cells to the IFN-γ-induced cell death but not to its cytostatic effects.

The cDNA carried by plasmid pTKO1- 229 was identified upon sequencing as a BamHI-HindIII fragment of human cathepsin D cDNA, which was present in the expression vector in the antisense orientation. The DNA probe, corresponding to fragment #229, hybridized as expected to a single endogenous 2.5 Kb mRNA, both in control and in the transfected HeLa cells. The steady state levels of cathepsin D sense mRNA were increased 3-4 fold by the IFN-γ treatment. In the pTKO1-229 transfected cells the DNA

probe also hybridized to the composite antisense RNA. The levels of antisense cathepsin D RNA were stimulated 5-fold in response to IFN-γ due to the presence of an ISRE enhancer element in the pTKO1 expression vector (not shown).

Cathepsin D is an aspartic protease that is found normally in lysosomes where it functions in protein catabolism. Yet, in some pathological situations it has been suggested that this protease can function in the cytosol, and its activity was associated with degenerative brain changes, muscular dystrophy and connective tissue disease pathology (Matus and Green (1987); Biochemistry, 26, 8083-8036). The present invention shows for the first time that the expression of this protease is indispensable for the execution of programmed cell death that is induced by IFN-γ and other cytokines (see below). Thus, cathepsin D joins the growing list of proteases that play a key role in different scenarios of programmed cell death.

The DNA sequence and amino acid sequence of cathepsin D are shown in Fig. 14 (Faust, P.L. *et al.* (1985) PNAS USA 82, 4910-4914).

XVIII. Anti-sense cathepsin D RNA and pepstatin A protect HeLa cells from IFN-γ-induced cell death.

(A) Experimental Procedure

1. Neutral-Red dye uptake assay

The HeLa cells were cultivated in 96-well microtiter plates at an initial number of 15,000 or 20,000 cells/well and were treated with either IFN-γ or anti-APO-1 antibodies, respectively, or were left untreated. Where indicated, pepstatin A (pepA) (Sigma) or DMSO were added to the culture medium. The culture medium and drugs were replaced every 3-4 days. Viable cells were stained with neutral-red (Sigma) as detailed before (Wallach D., J. Immunol.

132:2464-2469, 1984) The dye uptake was measured in quadruplicates at λ= 540 nm using an automated Micro-Elisa auto-reader.

2. RNA analysis

Total cellular RNA was extracted using Tri-ReagentTM (Molecular Research Center, Inc.). Samples of 20 μg total RNA were processed on Northern blots as previously described in detail (Yarden and Kimchi, Science 234: 1419-21, 1986). DNA fragments, used as probes, were purified from agarose gels with the Geneclean kit (BIO 101 Inc.). The fragments were labeled with 5 μCi of [-^{32}P]-dCTP (Amersham >3000 Ci/mmole), using a Random Priming kit (Boehringer).

3. Protein Analysis

Cells were extracted in RIPA (10 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate and 5 mM EDTA) containing a mixture of protease and phosphatase inhibitors (1mM PMSF, 4 μg/ml aprotenin, 100 μg/ml leupeptin, 1.5 μg/ml pepstatin A, 2 μg/ml antipain, 2 μg/ml chymostatin, 0.1mM NaVO3 and 0.1mM NaF). Protein concentration was determined using a Protein assay reagent (Bio-Rad). Aliquots of 300 g of the cell lysates were fractionated by SDS polyacrylamide gel electrophoresis (12%). The proteins were then electroblotted onto a nitrocellulose membrane and blots were incubated in blocking solution (10% skimmed milk and 0.05% Tween-20 (Sigma) in PBS) for 2 hours at room temperature, and then reacted with an antibody-containing solution for 18 hours at 4°C.

The washed membranes were incubated with peroxidase-conjugated second antibodies, either goat anti mouse IgG (IgG(H+L) chains, Jackson immuno Research Lab.) at a 1:10,000 dilution, or Protein A-conjugated to horse-radish peroxidase (Amersham) at a 1:10,000 dilution. Detection of the bound antibodies was carried out using ECL detection reagents (Amersham). The anti cathepsin-D monoclonal antibodies (EURO/DPC-U.K.) were used at 1:5 dilution; these antibodies recognize an epitope in the 30 Kd heavy chain. Polyclonal antibodies against the copper zinc superoxide dismutase (SOD)

were used at a 1:250 dilution. These antibodies were kindly provided by Y. Groner (Weizman Institute, Rehovot, Israel).

4. Transient transfections

Cathepsin D cDNA insert (2176 bp; SalI-EcoRI fragment containing the full length coding sequences and flanking non-coding regions (see Faust et al., 1985) was subcloned into the tetracycline-controlled expression vector (pSBC-TtA) (Dirks et al., Gene 128:247-249, 1993). The vector (40 μg) was transiently transfected into a HeLa cell clone (HtTA-1) that expresses the tetracycline transactivator gene, by the standard calcium phosphate technique ($2X10^5$ cells were seeded in 9cm plates 18-20 hours prior to transfection). An empty tetracycline-promoter containing vector was used as a control in the assays. In order to exclusively follow the transfected cells, these constructs were co-transfected with either the CMV-β-galactosidase gene (Clontech), or with the SEAP gene expressed from the SV40 promoter (the pSBC-2 vector) (Dirks et al., 1993). The molar ratio was 6:1 in favor of the tetracycline vectors. Each transfection was divided into two plates, one of which was immediately supplemented with tetracycline (1.5mg/ml). All the enzymatic activities were assessed 48h after transfections.

5. β-galactosidase staining and determination of SEAP activity

To detect LacZ expression, cells were fixed with 3% paraformaldehyde for 5 min., rinsed twice with PBS and stained for 3 hrs in X-Gal buffer containing 77mM Na2HPO4, 23mM NaH2PO4, 1.3mM MgCl2, 1mg/ml X-Gal, 3mM K3Fe(CN)6, and 3mM K4Fe(CN)6. The reaction was stopped by 70% ethanol. Photography was done under phase microscopy using Kodak Ektachrome 160T.

For the SEAP activity assay, the meduim of transfected cells was changed 5 hrs before the assay. Aliquots of 100 μl medium were removed from the transfected plates and heated at 65°C for 5 min. The medium was

then clarified by centrifugation at 14000 x g for 2 min. The medium aliqouts were adjusted to 1x SEAP assay buffer containing 2M diethanolamine pH 9.8, 1M MgCl2 and 20mM L-homoarginine. 20 ml of 120mM p-nitrophenylphosphate dissolved in water was then added to each mixture. The reaction mixtures were then incubated for 30 min. at 37°C. The hydrolysis of p-nitrophenylphosphate was measured at 405 nm.

(B) Results

First, the stable HeLa cells transfectants were tested for their growth sensitivity to IFN-γ. It was previously shown that parental HeLa cells displayed a biphasic pattern of response to IFN-γ, in which cells first ceased to proliferate but remained viable, followed by massive cell death with cytological characteristics of PCD (Deiss et al., Genes Dev. 9:15, 1995). One of the assays that was used to measure the anti-sense RNA mediated effects was based on neutral-red dye uptake into viable cells. In the absence of IFN-γ, both cell lines (DHFR and anti-cathepsin D-transfected cells) behaved the same and displayed identical growth curves. This suggested that the anti-sense RNA expression had no effects on the normal growth of cells (Fig. 41Aa). Also the extent of reduction in the neutral-red dye uptake during the first four days, corresponding to the cytostatic effects of IFN- (Deiss et al., 1995), was similar in both cell lines. This indicated that cathepsin D anti-sense RNA expression also did not interfere with the cell cycle inhibitory effects of the cytokine. The difference between the two cell populations became prominent later on during the IFN-γ-induced cell death phase. In the IFN-γ-treated DHFR-transfected cells, the dye uptake dropped from day 4 on (Figs. 41Aa, 41Ab). The microscopic observations confirmed that this was due to massive cell death that eliminated almost all the viable-adherent cells from the plates (Fig. 41Da). Death was significantly (but not completely) inhibited by the anti-sense cathepsin-D RNA expression, as shown by the sustained values of

the dye uptake (Fig. 41Aa). Each of the two anti-sense cathepsin D RNA expressing polyclonal populations displayed a significant increase in the fraction of cells that were stained by the viable dye during the IFN-γ-induced cell death phase (Fig. 41Ab).

Another way to measure protection from cell killing consisted of counting the number of colonies that were formed after releasing the cultures from long term treatment with the cytokine. The reduced susceptibility of the anti-sense transfected cells to cell killing by IFN-γ was manifested by a 1-2 logs increase in the number of cells that could form colonies, following the removal of IFN-g from treated cell cultures (Fig. 41B).

To further explore the participation of cathepsin D in the IFN-γ-induced PCD, the HeLa cells were incubated with pepstatin A, a specific inhibitor of aspartic proteases (Shields et al., Biochem. Biophys. Res. Comm. 177:1006, 1991). Due to the fact that cathepsin D is the major intracellular aspartic protease in cells, the outcome of the intracellular effects of this penta-peptide are commonly attributed to the specific inhibition of cathepsin D activity. Pepstatin A was added to the culture medium at a final concentration of 10^{-4}M in 0.2% DMSO, in accordance with previous reports whereby similar incubation protocols led to effective intracellular concentrations of the drug (Shields et al., 1991). Pepstatin A had no effect on growing HeLa cells that were not treated with IFN-γ. Addition of pepstatin A to the IFN-γ-treated DHFR-transfected cells inhibited, to some extent, the killing process, as reflected by the elevated values of neutral-red dye uptake (Fig. 41C). The highest values of dye uptake that could be measured in the presence of IFN-γ were obtained by applying the pepstatin A to the anti-sense cathepsin D RNA expressing cells (Fig. 41C).

Microscopic examination of the IFN-γ-induced cell cultures that were protected by the double treatment (anti-sense RNA + pepstatin A) revealed

that the majority of cells displayed the normal adherent phenotype, whereas only about 20% of the cells had a round- apoptotic morphology (Fig. 41Db).This further indicated that the combined reduction of both expression and activity of this endoprotease was most effective in protecting cells from IFN-γ-induced cell death. In summary, the anti-sense RNA and pepstatin A data suggest an active role for cathepsin D in the IFN-γ-mediated PCD.

XIX. Regulation of cathepsin D protein expression and processing during the IFN-γ-induced PCD.

The effects of IFN-γ on the expression and processing of cathepsin D protein were then analyzed on immunoblots. A typical change in the relative abundance of the different cathepsin D forms was detected in the treated cells (Figs. 42A and 42B). The 48 Kd form of cathepsin D, usually detected in trace amounts in untreated HeLa cells, gradually accumulated to high levels between days 4-7 of IFN-γ-treatment. In contrast, the steady state levels of the 30 Kd form were not increased (Fig. 42B) and in some experiments were even reduced at the late time points (Fig. 42A).

The 48 Kd cathepsin D is a proteolytic active, single chain form often found in pre-lysosomal vesicles. It is normally targeted to lysosomes whereby it is further processed into the double-chain form (30 and 14 Kd) of the enzyme (see the scheme in Fig. 42C- note that the monoclonal antibodies used in Fig. 42 are directed against an epitope in the heavy 30 Kd chain). The unusual accumulation of the 48 Kd form, therefore, suggested that the normal processing of the protease was interrupted during the IFN-γ-mediated cell death. In addition, on days 5-7 of IFN-γ treatment, the levels of the 48Kd precursor were 8-10 fold higher than the levels of the 30 Kd form before treatment (Fig. 42A). The increased steady state levels of cathepsin D proteins could result, at least partially, from the RNA elevations.

It is noteworthy that in some, but not all experiments, the intracellular levels of the 52 Kd prepro-cathepsin D form increased as well after IFN-γ treatment of the parental cells (Fig. 42A). Traces of the 52 Kd form were also found in the culture medium, but no effects of IFN-γ on the levels of this secreted form were detected (not shown).

The prominent IFN-γ-mediated elevation of cathepsin D protein and the accumulation of the intermediate forms were both prevented in the HeLa polyclonal cell population expressing the anti-sense RNA (Fig. 42B; the calculated values were 8.2 and 1.1 fold increase by IFN- in the cathepsin D protein forms for DHFR and anti-cathepsin D transfectants, respectively). A few independently generated anti-sense expressing polyclonal populations were examined and none of them displayed elevated levels of cathepsin D in response to IFN-γ. These findings, therefore, confirmed that the large excess of anti-sense over sense RNA during the IFN-γ selection effectively reduced the total levels of cathepsin D protein, as was expected. The question as to why the residual levels that continued to be expressed in these IFN-γ-treated cells, did not accumulate as intermediate forms of cathepsin D, is still open.

XX. Cathepsin D aspartic protease mediates the APO-1/Fas and the TNF-α-induced PCD.

The question of whether cathepsin D protease is also involved in other apoptotic systems, triggered by the activation of cell surface receptors that differ from the IFN-γ receptors was also studied. The different HeLa cell transfectants were treated with the agonistic anti-APO-1 monoclonal antibody, in order to determine whether cathepsin D mediates the Fas/APO-1-induced apoptosis. The parental and DHFR-transfected cells were efficiently killed by anti-Fas/APO-1 antibodies. Cell death exhibited features characteristic of apoptosis, similar to the IFN-γ effects. By 40 hours, about 70% of the cells

rounded up and detached from the plates (not shown) and the uptake of neutral red dye was reduced accordingly (Fig. 43A). The killing required a short pretreatment of the cells with a low dosage of IFN-γ (50U/ml), which had no effect by itself on cell viability. The low dosage of IFN-γ sensitized the cells to killing by the agonistic antibody, due to elevation of the Fas/APO-1 expression. Expression of anti-sense cathepsin D RNA, or alternatively the addition of pepstatin A to the culture meduim of the DHFR-transfected cells, substantially suppressed the Fas/APO-1-mediated cell death resulting in an increased fraction of viable cells (Fig. 43A). The latter indicated that cathepsin D is essential for the Fas/APO-1-induced PCD.

It was also found that pepstatin A interfered with the apoptotic process that is triggered in U937 histiocytic lymphoma cells by tumor necrosis factor-α (TNF-α). The killing in this system was very rapid, and characterized by typical nuclear changes such as chromatin condensation followed by its fragmentation. DAPI staining of U937 nuclei indicated that 6 hours after TNF-α administration approximately a third of the cell population already contained nuclei with typical fragmented chromatin (Fig. 43B). Addition of pepstatin A to the culture showed a significant reduction in the number of fragmented nuclei (Fig. 43B). Interestingly, the earlier step of chromatin condensation seemed less susceptible to the effect of pepstatin A. These data indicated that cathepsin D endoprotease also mediates some critical steps along the apoptotic pathway, which leads to U937 cell death.

Examination of the pattern of cathepsin D expression in the TNF-α-treated U937 cells revealed that it shared a few common features with the HeLa cell system. The total levels of cathepsin D proteins were significanly increased. Moreover, the proteolytic active 48 Kd intermediate form accumulated in these TNF- -treated U937 cells, indicating that again the processing into the double chain form was interrupted (Fig. 42D). Yet, in

contrast to the HeLa cell system, this conversion was not completely blocked and a mild increase in the 30 Kd form was detected as well. These data suggest a common pattern of changes in the expression/processing of cathepsin D protein in a few apoptotic systems.

XXI. Ectopic expression of cathepsin D is not compatible with cell viability.

The outcome of overexpression of cathepsin D was directly measured in HeLa cells by co-transfections with the lacZ gene used as a marker of gene expression. Cathepsin D was driven by the tetracycline-repressible promoter (Gossen and Bujard, PNAS 89:5547-5551, 1992) and the -galactosidase gene was driven by the CMV constitutive promoter. The morphology of lacZ containing blue cells was determined 48 hours post transfection, in cultures which were maintained in the absence of tetracycline to allow cathepsin D transcription/translation. It was found that 70% of the lacZ containing cells displayed a round apoptotic phenotype upon co-transfections with cathepsin D, whereas co-transfections with the control tetracycline vector displayed a background of less than 20% apoptotic cells (Figs. 44A, 44B, 44C).

In order to further quantitate the effects of ectopic expression of cathepsin D on cells, in a second independent approach, co-transfections were performed with vectors expressing the secreted alkaline phosphatase (SEAP) instead of lacZ. In these experiments the outcome of tetracycline withdrawal on SEAP activity, released by transfectants carrying the cathepsin D gene was measured. It was found that the activation of cathepsin D by tetracycline withdrawal significantly reduced the SEAP activity secreted into the culture medium around 48 hours post-transfection, as compared to the values obtained from the same population maintained in the presence of tetracycline (Fig. 44D). In contrast, tetracycline withdrawal had no effect on SEAP

activity released by control cultures which were co-transfected with the empty vector.

XXII. DAP kinase expression in metastatic cell lines

(A) Experimental Procedure

(A_1) Transfections

Transfections were performed by the standard calcium phosphate technique.

(A_2) *In vitro* immune complex assay for DAP-kinase

Immunoprecipitation of recombinant DAP-kinase protein from 1 mg total extract of transfected cells was done with 20 µl anti-FLAG M2 gel (IBI, Kodak) in 200 µl of PLB supplemented with protease and phosphatase inhibitors for 2h at 4°C. Following three washes with PLB, the immuno-precipitates were washed once with reaction buffer (50 mM Hepes pH 7.5, 8 mM $MgCl_2$, 2 mM $MnCl_2$ and 0.1 mg/ml BSA). The proteins bound to the beads were incubated for 15 min. at 25°C in 50 µl of reaction buffer containing 15 µCi[γ^{-32}P] ATP (3 pmole), 50 mM ATP, 5 µg MLC (Sigma) and 1 µM bovine calmodulin (Sigma), and 0.5 mM $CaCl_2$. Protein sample buffer was added to terminate the reaction, and after boiling the proteins were analyzed on 11% SDS-PAGE. The gel was blotted onto a nitrocellulose membrane and ^{32}P labeled proteins were visualized by autoradiography.

(A_3) DAPI staining of nuclei before and after treatment with TNF-α.

Exponentially growing cells were treated with a combination of murine TNF-α (100 ng/ml; R&D Systems, Minneapolis) and cycloheximide (5 µg/ml; Sigma) (right panels marked by +), or with cycloheximide along (left panels marked by -). DAPI staining was performed after 6 hours. The cells were plated on glass cover-slips (13 mm diam.), 20,000 cells/well in 1 ml medium within a 24-wells plate. Cells were washed twice with PBS,

fixed and permeabilized simultaneously. This was carried out by incubating the cover-slips for 5 min. in a mixture of 3% paraformaldehyde and 0.3% Triton X-100 in PBS, and then incubating with 3% paraformaldehyde alone for additional 20 min. The cells were washed three times in PBS and then incubated in blocking solution (5% normal goat serum and 1% BSA in PBS) for 60 min. DAPI (0.5 μg/ml; Sigma) was added at this stage.

(B) Results

DAP-kinase protein expression was examined in two sets of high- and low-metastatic cell lines selected from the mouse Lewis and CMT64 lung carcinoma cells. Intriguingly, the two different high-metastatic cell lines did not express DAP-kinase mRNA (not shown) or protein, whereas their low-metastatic cell counterparts were DAP-kinase positive (see Fig. 17 for A9-F and D122 sublines of the murine Lewis lung carcinona, displaying low and high metastatic capabilities, respectively). The goal was then to introduce into the high-metastatic D122 cells a functional DAP-kinase and to test the influence of this genetic manipulation on the tumorigenic and metastatic potential of these aggressive tumor cells.

FLAG-tagged wild type DAP-kinase, cloned in pCDNA3 was transfected into the D122 cells by the calcium phosphate co-precipitation technique. An empty pCDNA3 vector was used for the control transfections. Several stable DAP-kinase positive clones were isolated which were classified into low (6-DAP-kinase), low-mid (48-DAP-kinase), mid (28-DAP-kinase) and high (42-DAP-kinase) expressing cells (Fig. 17; the A9-F low-metastatic cells were used as a reference). Three clones transfected with the control vector, did not express DAP-kinase, as expected (Fig. 17). Next, an *in vitro* immune complex assay for DAP-kinase was performed following immuno-precipitation by the anti-FLAG antibodies, in order to test whether the exogenously expressed kinase is active. It was found, by using the myosin light chain (MLC) protein as a substrate, that the DAP-kinase protein expressed from the transfected vector was catalytically active (Fig. 18).

The growth rate in culture of the DAP-kinase positive transfectants, in medium containing 10% or 1% fetal calf serum (FCS), was similar to that of the control and the parental clones (Fig. 19). A single exception was the 42-DAP-kinase clone which at the high serum concentration grew slightly slower (two fold increase in the doubling time) due to some disruptions of

cytokinesis. DAPI staining of the nuclei of the 42-DAP-kinase cells, growing in 10% FCS-containing medium, showed that the frequency of the fragmented nuclei was below 0.1% (Fig. 27), thus indicating that DAP-kinase by itself, even in the high-expressing stable clone, did not trigger apoptosis. Altogether, it was concluded that the restoration of DAP-kinase expression had either null or subtle effects on the continuous growth of the cells in culture.

XXIII. *In vivo* activity of DAP-kinase transfected cells

(A) Experimental Procedures

(A_1) **Experimental metastasis.**

The different D122-transfected clones were injected into tail veins of 10-12 week old C57BL/6 female mice ($5x10^5$ cells per mouse). Mice were sacrificed 30-32 days later, and their lungs were removed, weighed and fixed in Bouin's solution. The number of metastatic nodules were determined by counting surface nodules under a binocular.

(A_2) **Local tumor growth assay**

The different D122-transfected clones were injected into the footpads of C57BL/6 mice (10-12 week old females); ($2x10^5$ cells per mouse). Diameters of tumor bearing feet were measured using calipers every 1-3 days. When tumor diameter reached 8-9 mm, tumor bearing feet were amputated below the knee and the day of death resulting from spontaneous lung metastasis was scored for each individual mouse. In a few cases, the tumor cells were recovered in culture from dissected lung nodules and grown, like all the D122 transfectants, in medium containing 10% fetal calf serum supplemented with G418 (800 μg/ml).

(B) Results

The tumorigenic and metastatic potential of DAP-kinase transfected cells were assayed in mice, where they may be exposed to a variety of death-inducing signals. For example, in the blood stream, the invading tumor cells must resist programmed cell death that is induced by interactions with cytotoxic T lymphocytes, natural killer cells, and macrophages, and with the cytokines which these hematopoietic cells secrete (e.g., IFN's, TNF, IL-1β). They must also resist the apoptotic cell death induced by nitric oxide anions produced by the endothelial cells, and withstand mechanical shearing forces caused by the hemodynamic turbulence. Moreover, during the intravasation or extravasation processes, and during the growth in a foreign hostile micro environment, locally produced inhibitory cytokines (e.g., TGF-β) or loss of cell-matrix interactions (e.g., detachment from the basement membranes) also trigger apoptotic cell death.

The injections into the C57BL/6 syngeneic mice consisted of two different experimental systems and were repeated in three independent experiments. One group received intrafootpad injections ($2x10^5$ cells per injection) in order to follow the local tumor growth. The second group received intravenous injections ($5x10^5$ cells per injection) in order to follow experimental metastases in the lungs.

It was found that the growth of the local tumor in the footpads was significantly delayed as compared to the parental and the G-418 resistant control clones, and that the length of the delay was directly propotional to the levels of the ectopically expressed DAP-kinase (Fig. 20; a lag of 10 days was characteristic of the mid-expressing clone (28-DAP-kinase) and a delay of more than 50 days characterized the high-expressing clone (42-DAP-kinase).

To examine the effect of DAP-kinase on the experimental metastasis, the lungs were examined 30-32 days after the intravenous injections. Metastasis was strongly suppressed, as measured by the average lung weight

and by the mean number of metastatic lesions (Figs. 21, 22). The experimental metastasis assay was much more sensitive to DAP-kinase expression than the local tumor growth assay. In the lung assay even the low and low-mid expressing clones (6-DAP-kinase; 48-DAP-kinase, respectively) displayed almost maximal reduction of lung weight and of the number of metastatic lesions, while the effects on the local tumor growth were very mild or even undetectable in the low-mid and low expressing clones, respectively.

XXIV. Loss and restoration of metastatic suppression

(A) Results

Spontaneous metastasis eventually appeared in the lungs of all the experiments that used the 28-DAP-kinase clone, and in some but not all of the 42-DAP-kinase clone injections, after the tumor bearing legs were amputated. It was interesting to test whether the mid- and high- DAP-kinase expressing clones, which eventually grew in the mice footpads (after the lag period), and which were capable of generating spontaneous metastases in the lungs after the amputation of the tumor bearing legs, were selected *in vivo* for loss or inactivation of the transfected gene. It was found that the cells which were released in cultures from the lungs of mice that received intrafootpad injections of the 28-DAP-kinase and 42-DAP-kinase clones, expressed traces or even undetectable levels of exogenous DAP-kinase (Fig. 23, lanes 1,2; Fig. 24, lanes 1,2; Fig. 25, lane 1). A strong selection for attenuation of DAP-kinase ectopic expression, therefore occurred *in vivo*, probably during the lag period before the tumor took in the footpads.

It was possible to restore the full expression capacity of the transfected gene, in clone 28-DAP-kinase that underwent the in vivo selection, by treating the cells in culture with the demethylating agent, 5-aza-2'-deoxycytidine (Fig. 25, lanes 1,2). The restoration was transient and the DAP-kinase levels returned to their suppressed levels a few passages after the removal of the

drug (Fig. 25, lanes 3,4). No effects were detected in the parental D122 cells that continued to lack DAP-kinase expression after similar treatments with 5-aza-2'-deoxycytidine (not shown). The *in vivo* selection for attenuated expression of the transfected DAP-kinase gene, therefore occurred by DNA methylation, an epigenetic mechanism that is frequently used by human tumors to turn off various tumor suppressor genes including the endogenous DAP-kinase gene in bladder carcinoma cells. This attenuation did not occur in the 28-DAP-kinase transfectants that were released from experimental metastasis, i.e., from the very few small metastases that were present in the lungs after the intravein injections. As shown in Fig. 23 (lanes 3,4), the DAP-kinase levels were identical to those detected in the original injected clone. This is consistent with the strong suppression of the metastatic phenotype described above.

XXV. Response of DAP-kinase transfected cells to apoptotic stimuli

(A) Experimental Procedure

(A_1) *In situ* TUNEL staining-apoptic index.

Fragments of mice footpads were fixed for 12 hours in 4% buffer formaldehyde (Frutarom), embedded in paraffin, and sectioned (4 μm thick). TUNEL assays on these sections (peroxidase staining of fragmented DNA and counterstaining of the sections by methyl green dye) was performed according to manufacturer's instructions (ApopTag® Plus Peroxidase Kit; Oncor, Gaithersburg). Six different sections were scored; in each case 500-1000 tumor cells were counted and the mean apoptic index was calculated. The mean values were $6.3\% \pm 1.13$ and $1.9\% \pm 0.35$ for 42-DAP-kinase and 4-cont., respectively. The difference was significant at $P << 0.001$.

(A_2) Soft agar-anchorage independent growth.

The different clones were cultured in 0.33% soft agar (Bacto-agar; Difco) at an initial cell number of $5x10^3$ cells per 6 cm plate, on top of a layer containing 0.5% agar. (A) The diameters of the clones that appeared on day 7 were measured under a light microscope. Values are the mean colony diameter of 100 clones from each group ± SD. The difference between the controls (e.g., 18-cont.) and the DAP-kinase-transfectants (e.g., 1-DAP-kinase) was significant at $P<<0.001$. (B) Microscopy of the clones cultured in soft agar for seven days as in (A), comparing the parental D122 cells (left: a,c) to DAP-kinase-42 cells (right: b,d). The bars correspond to 350 mm in the upper panels (a,b) and to 80 mm in the lower panes (c,d).

(B) Results

In order to find out whether the anti-tumorigenic and anti-metastatic effects of DAP-kinase resulted from the increased sensitivity of the cells to apoptotic signals, in situ TUNEL staining was performed on histological sections of the mice footpads, five days post-injection. The staining illustrated that the apoptotic index in the slow growing local tumors formed by the DAP-kinase-transfected cells, was significantly higher than the value measured in the tumor mass formed by the control clone (Fig 26). The calculated values were 6% and 2%, respectively, which should reflect a tremendous difference in total cell death, in view of the rapid elimination of apoptotic cells by macrophages and by neighbor cells. The in situ staining provided the first hint implicating the DAP-kinase gene in augmenting the threshold sensitivity of the tumor cells to different apoptotic signals.

To further address this issue directly, some more defined types of apoptotic stimuli were applied in culture, one of which was the death-inducing cytokine-TNF-α. It was found that clone 42-DAP-kinase displayed higher sensitivity to the TNF- -induced cell death, as measured by DAPI staining of the nuclei a few hours after administration of the cytokine

(Fig. 27; 28). Interestingly, this increased sensitivity to the apoptotic effects of TNF-α was lost following the *in vivo* selection of clone 42-DAP-kinase for attenuated DAP-kinase expression, thus linking more directly, within the same genetic background, DAP-kinase expression and apoptosis (Fig. 28).

Other types of apoptotic stresses were imposed on the cells by transferring them into soft agar, where their anchorage-independent growth could be tested. In contrary to the large colonies formed in soft agar by the parental D122 cells and the control clones, the DAP-kinase transfectants formed abortive small colonies in which most of the cells died (Figs. 29,30). A reversion to large colonies was obtained when the aforementioned *in vivo* selected 42-DAP-kinase clone, which displays the attenuated DAP-kinase expression, was tested (data not shown). It is therefore concluded that cell death which is induced by detachment from the extracellular matrix, or by other yet unidentified mechanisms that may operate during the loss of anchorage-dependent growth, depends on the presence of functional DAP-kinase. This is an example of non-cytokine induced programmed cell death.

XXVI. Non-cytokine inducers of programmed cell death

In addition to the programmed cell death induced by detachment from the extracellular matrix, another example of non-cytokine induced programmed cell death occurs in mature neuronal cells which undergo apoptotic cell death in response to a variety of stress conditions, including lack of neurotrophic factors, anoxia, excitotoxicity, traumatic injury or neurodegenerative disorders. When death of post-mitotic neurons occurs in the CNS of a mammalian organism, it is detrimental since there is no regeneration of this tissue. The outcome of such lesion would then be a permanent loss of function.

In order to determine the effect of DAP molecules on non-cytokine induced programmed cell death in neurons, primary cultures of

hippocampal neurons may be prepared from 18 day old rat embryos. These cell cultures can serve as a model for certain scenarios where cell death is triggered.

There are several advantages in using a hippocampal cell culture: (1) It is a homogenous culture of neurons with regard to their cell surface receptors for neurotransmitters; (2) Hippocampal neurons are most susceptible to induction of cell death by various conditions: excitotoxicity (e.g. glutamate), anoxia, oxygen radicals, deprivation of trophic factors. For example, to test the role of DAP-kinase in these systems, the catalytically inactive mutant version, K42A, may be used. An adenovirus-mediated gene transfer system may be used to introduce the K42A DAP-kinase into the quiescent nerve cells, and the apoptotic responses to the various non-cytokine inducers may be assesed.

Other non-cytokine inducers of programmed cell death in mammalian cells in general include radiation (both γ and UV), the p53 gene, etc.

While the present invention has been described in terms of several preferred embodiments, it is expected that various modifications and improvements will occur to those skilled in the art upon consideration of this disclosure.

The scope of the invention is not to be construed as limited by the illustrative embodiments set forth herein, but is to be determined in accordance with the appended claims.

CLAIMS:

1. Use of a therapeutically effective amount of an expression vector comprising a DNA sequence capable of inducing programmed cell death, in the preparation of a pharmaceutical composition for use in the treatment of a disease or a disorder associated with metastasizing pathological cell growth, said DNA sequence being selected from the group consisting of:

(a) a DNA sequence expressed in cells, the expression product of which is involved in programmed cell death;

(b) a DNA sequence, other than the DNA defined under (a), which encodes the same expression product encoded by the DNA sequence defined in (a);

(c) a modified DNA sequence of (a) or (b) in which one or more nucleic acid triplets has been added, deleted, or replaced, the protein or polypeptide encoded by the modified DNA sequence mediating the programmed cell death similarly to the protein or polypeptide encoded by said gene as defined under (a) or (b); and

(d) fragments of any of the DNA sequences of (a), (b) or (c), encoding a protein or a polypeptide having said biological activity.

2. A use according to Claim 1, wherein said DNA sequence is a nucleic acid sequence expressed in cells, the expression product of which is involved in programmed cell death, being one of the following:

(viii)SEQ. ID. NO.:1;

(ix)SEQ. ID. NO.:2;

(x)SEQ. ID. NO.:3;

(xi)SEQ. ID. NO.:4;

(xii)SEQ. ID. NO.:5;

(xiii)SEQ. ID. NO.:7; or

(xiv)a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 201-203 and ending at the triplet 3018-3020 of the sequence depicted in Fig. 15 (SEQ. ID. NO.:8).

3. A use according to claim 2 wherein said DNA sequence is SEQ. ID. NO.:3.

4. A use according to Claim 1, wherein said DNA sequence is a DNA molecule encoding the same protein or polypeptide encoded by any one of the nucleic acid sequences defined in Claim 2.

5. A use according to Claim 1, wherein said DNA sequence is a nucleic acid sequence as defined in claim 2 in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the sequence having essentially the same biological activity as that encoded by any one of the DNA molecules of claim 2.

6. A use according to Claim 1, wherein said DNA sequence is a fragment of a nucleic acid sequence as defined in claim 2 encoding a protein or polypeptide retaining a biological activity present in the protein or polypeptide encoded by said nucleic acid sequence as defined in claim 2.

7. A use according to Claim 1, wherein said pharmaceutical composition is administered together with a cytokine.

8. A method of determining the prognosis of a metastatic disease comprising:

(a) obtaining a sample from an individual suffering from said disease, said sample being a tissue section or either genomic DNA or mRNA obtained from cells, or cDNA produced from said mRNA;

(b) adding to said sample one or more nucleic acid probes, said one or more probes comprising a sequence selected from the group consisting of:

(i) a DNA sequence expressed in cells, the expression product of which is involved in programmed cell death;

(ii) a modified DNA sequence of (i) in which one or more nucleic acid triplets has been added, deleted, or replaced, said modified DNA sequence retaining the capability of hybridizing with the sequence of (i);

(iii) a sequence which is an antisense to the entire or part of the DNA sequence of (i) or (ii); and

(iv) an RNA sequence which is complementary to the DNA sequence of (i), (ii) or (iii) ;

(c) providing conditions for hybridization between the one or more probes and said sample; and

(d) determining on the basis of said hybridization whether a gene involved in programmed cell death is associated with said metastatic disease, an absence of hybridization indicating a lack of said gene, and an abnormal hybridization indicating a possible inactivation of said gene.

9. A method according to Claim 8 wherein said one or more DNA probes comprise a complete or partial sequence of a DNA sequence selected from the group consisting of:

(i)SEQ. ID. NO.:1;

(ii)SEQ. ID. NO.:2;

(iii)SEQ. ID. NO.:3;

(iv)SEQ. ID. NO.:4;

(v)SEQ. ID. NO.:5;

(vi)SEQ. ID. NO.:7; or

(vii)a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 200-202 and ending at the triplet 3017-3019 of the sequence depicted in Fig. 15 (SEQ. ID. NO.:8).

10. A method of determining the prognosis of a metastatic disease comprising:

(a) obtaining a sample from an individual suffering from said disease, said sample being a cell extract or a tissue section;

(b) adding to said sample one or more specific antibodies capable of binding a protein encoded by the DNA as defined in Claims 1-3;

(c) providing conditions for the binding of said proteins in said sample by said antibodies; and

(d) determining on the basis of the binding of said proteins whether a protein involved in programmed cell death is associated with said metastatic disease, a lack of binding indicating an absence of said protein, and an abnormal binding indicating a possible modification of said protein.

11. A DNA molecule comprising a sequence selected from the group consisting of:

(a) a gene whose expression product is necessary for the mediation of cytokine-induced programmed cell death; and

(b) a DNA sequence encoding the same protein or polypeptide encoded by the gene defined in (a);

wherein said DNA molecule has undergone a mutation resulting in the product of said mutated molecule having increased cytokine-induced programmed cell death promoting activity.

12. A DNA sequence comprising a nucleic acid sequence beginning at position 1767 and ending at position 2529 of the sequence depicted in Fig. 15.

13. Use of a therapeutically effective amount of pepstatin A in the preparation of a pharmaceutical composition for use in the treatment of cathepsin D-related programmed cell death.

14. Use of a therapeutically effective amount of an expression vector comprising a DNA sequence capable of promoting non-cytokine-induced programmed cell death, in the preparation of a pharmaceutical composition useful in the treatment of a disease or a disorder associated with uncontrolled

pathological cell growth, said DNA sequence being selected from the group consisting of:

(a) a DNA sequence expressed in cells, the expression product of which is involved in non-cytokine induced programmed cell death;

(b) a DNA sequence, other than the DNA defined under (a), which encodes the same expression product encoded by the DNA sequence defined in (a);

(c) a modified DNA sequence of (a) or (b) in which one or more nucleic acid triplets has been added, deleted, or replaced, the protein or polypeptide encoded by the modified DNA sequence mediating the programmed cell death similarly to the protein or polypeptide encoded by said gene as defined under (a) or (b); and

(d) fragments of any of the DNA sequences of (a), (b) or (c), encoding a protein or a polypeptide having said biological activity.

15. A use according to Claim 14, wherein said DNA sequence is a nucleic acid sequence expressed in cells, the expression product of which is involved in non-cytokine induced programmed cell death, being one of the following:

(i) SEQ. ID. NO.:1;

(ii) SEQ. ID. NO.:2;

(iii) SEQ. ID. NO.:3;

(iv) SEQ. ID. NO.:4;

(v) SEQ. ID. NO.:5;

(vi) SEQ. ID. NO.:7; or

(vii) a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 201-203 and ending at the triplet 3018-3020 of the sequence depicted in Fig. 15 (SEQ. ID. NO.:8).

16. A use according to claim 15 wherein said DNA sequence is SEQ. ID. NO.:3.

17. A use according to Claim 14, wherein said DNA sequence is a DNA molecule encoding the same protein or polypeptide encoded by any one of the nucleic acid sequences defined in Claim 15.

18. A use according to Claim 14, wherein said DNA sequence is a nucleic acid sequence as defined in Claim 15 in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the sequence having essentially the same biological activity as that encoded by any one of the DNA molecules of claim 15.

19. A use according to Claim 14, wherein said DNA sequence is a fragment of a nucleic acid sequence as defined in claim 15 encoding a protein or polypeptide retaining a biological activity present in the protein or polypeptide encoded by said nucleic acid sequence as defined in claim 15.

20. A use according to Claim 14 wherein said disease is cancer.

21. A DNA molecule comprising a sequence selected from the group consisting of:

(a) a gene whose expression is necessary for the mediation of non-cytokine-induced programmed cell death; and

(b) a DNA sequence encoding the same protein or polypeptide encoded by the gene defined in (a);

wherein said DNA molecule has undergone a mutation resulting in the product of said mutated molecule having increased cytokine-induced programmed cell death promoting activity.

22. Use of a therapeutically effective amount of an expression vector comprising a DNA sequence capable of inhibiting non-cytokine induced programmed cell death, in the preparation of a pharmaceutical composition useful in the treatment of a disease or a disorder associated with non-cytokine induced programmed cell death, said DNA sequence being selected from the group consisting of:

(a) a sequence which is an antisense to the entire or part of the a DNA molecule expressed in cells, the expression product of which is involved in non-cytokine induced programmed cell death, said antisense being capable of inhibiting the expression of said DNA molecule; and

(b) a modified DNA sequence of a DNA molecule as defined in (i) in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the modified sequence having dominant negative effect manifested by the ability of said protein or polypeptide to inhibit said programmed cell death; and

(c) an inhibitor or antagonist of any of the proteins or polypeptides encoded by the DNA sequences defined in Claim 15.

23. A use according to Claim 22, wherein said DNA sequence is a sequence which is an antisense molecule complementary in sequence to the mRNA transcribed from the entire or part of a nucleic acid sequence as defined in claim 15 and capable of inhibiting the expression of said sequence.

24. A use according to Claim 22, wherein said DNA sequence is a modified DNA sequence of any one of the sequences as defined in Claim 15 in which one or more nucleic acid triplets has been added, deleted or replaced, the protein or polypeptide encoded by the modified sequence having dominant negative effect and being capable of inhibiting the function of the protein or polypeptide encoded by any one of said sequences defined in Claim 15.

25. A use according to Claim 22 wherein said disease is Alzheimer's disease or Parkinson's disease.

26. A method of determining the prognosis of a disease associated with uncontrolled pathological cell growth comprising:

(a) obtaining a sample from an individual suffering from said disease, said sample being a tissue section or either genomic DNA or mRNA obtained from cells, or cDNA produced from said mRNA;

(b) adding to said sample one or more nucleic acid probes, said one or more probes comprising a sequence selected from the group consisting of:

(i) a DNA sequence expressed in cells, the expression product of which is involved in non-cytokine induced programmed cell death;

(ii) a modified DNA sequence of (i) in which one or more nucleic acid triplets has been added, deleted, or replaced, said modified DNA sequence retaining the capability of hybridizing with the sequence of (i);

(iii) a sequence which is an antisense to the entire or part of the DNA sequence of (i) or (ii); and

(iv) an RNA sequence which is complementary to the DNA sequence of (i), (ii) or (iii) ;

(c) providing conditions for hybridization between the one or more probes and said sample; and

(d) determining on the basis of the hybridization whether a gene involved in non-cytokine induced programmed cell death is associated with said disease, an absence of hybridization indicating a lack of said gene, and an abnormal hybridization indicating a possible inactivation of said gene.

27. A method according to Claim 26 wherein said one or more DNA probes comprise a complete or partial sequence of a DNA sequence selected from the group consisting of:

(i) SEQ. ID. NO.:1;

(ii) SEQ. ID. NO.:2;

(iii) SEQ. ID. NO.:3;

(iv) SEQ. ID. NO.:4;

(v) SEQ. ID. NO.:5;

(vi) SEQ. ID. NO.:7; or

(vii) a DNA sequence comprising a coding sequence beginning at the nucleic acid triplet at position 200-202 and ending at the triplet 3017-3019 of the sequence depicted in Fig. 15 (SEQ. ID. NO.:8).

28. A method of determining the prognosis of a disease associated with uncontrolled pathological cell growth comprising:

(a) obtaining a sample from an individual suffering from said disease, said sample being a cell extract or a tissue section;

(b) adding to said sample one or more specific antibodies capable of binding a protein encoded by the DNA as defined in Claims 14-16;

(c) providing conditions for the binding of said proteins in said sample by said antibodies; and

(d) determining on the basis of the binding of said proteins whether a protein involved in non-cytokine induced programmed cell death is associated with said metastatic disease, a lack of binding indicating an absence of said protein, and an abnormal binding indicating a possible modification of said protein.

29. A use according to any of claims 14-20 or 22-24 wherein said non-cytokine induced programmed cell death is induced by the loss of anchorage-dependent growth of said cells.

30. A use according to any of claims 14-20 or 22-25 wherein said non-cytokine induced programmed cell death is induced in neuronal cells by glutamate.

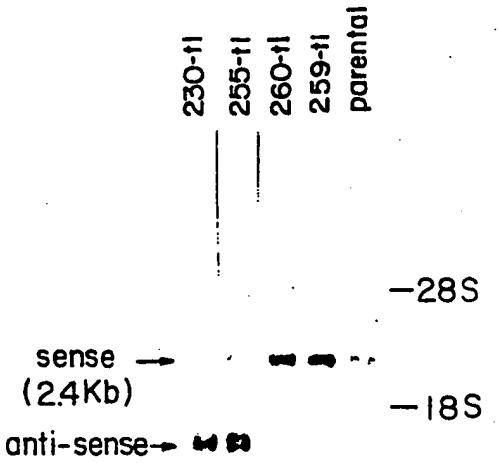

FIG. 1A

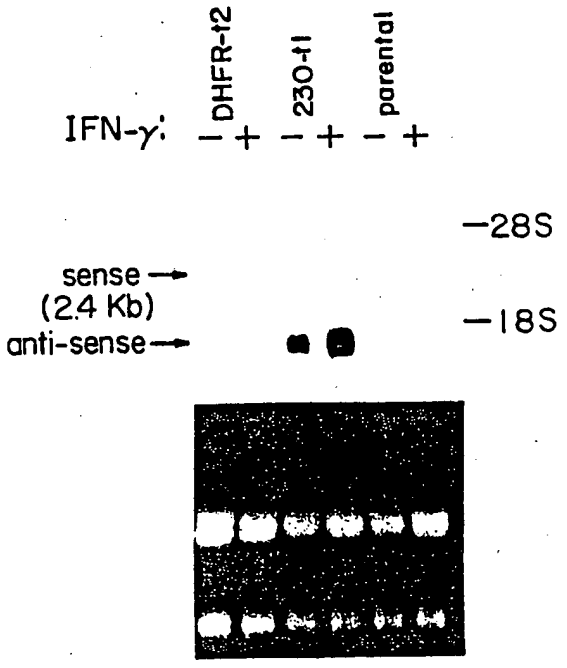

FIG. 1B

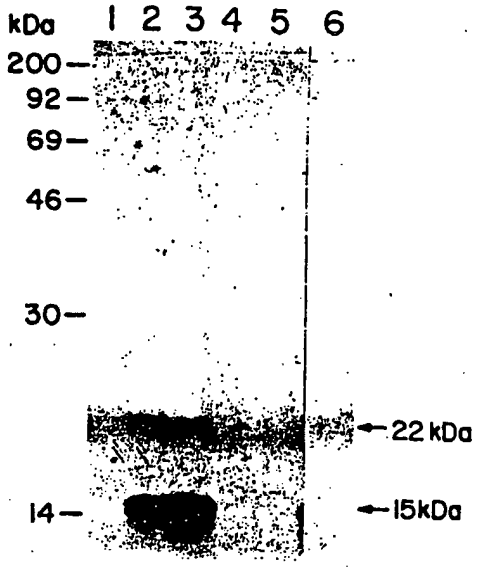

FIG. 1C

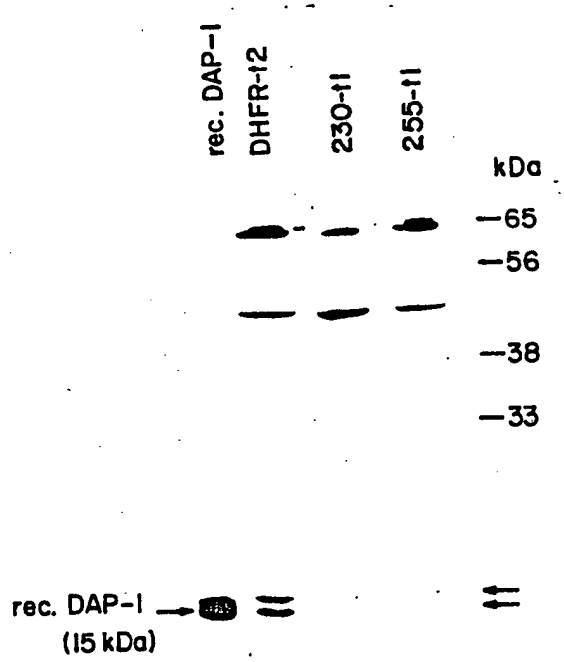

FIG. 1D

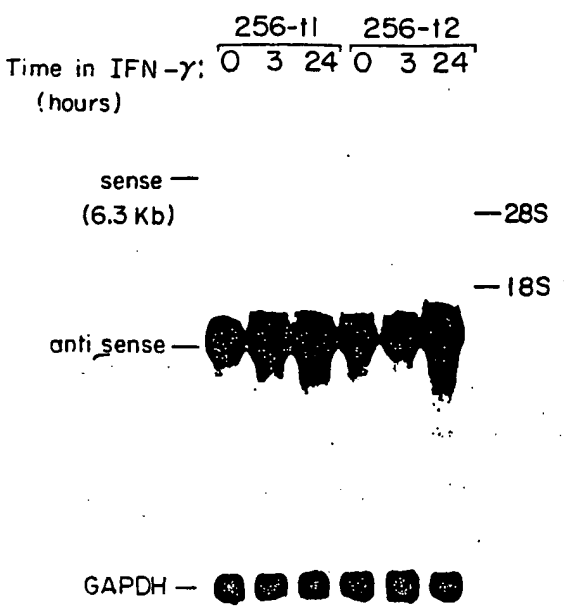

FIG. 2A

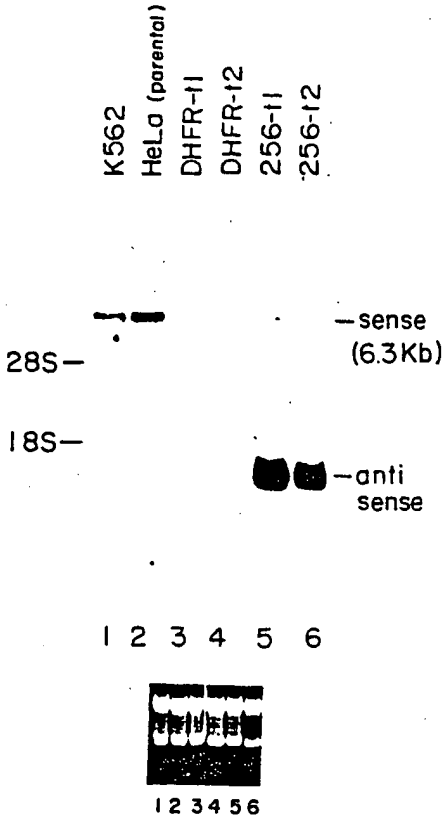

FIG. 2B

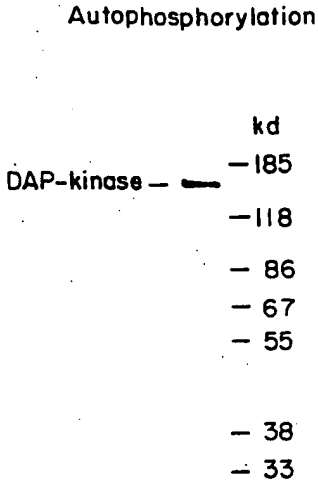

FIG. 2C

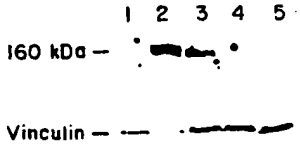

FIG. 2D

FIG. 3A(a) FIG. 3A(c)

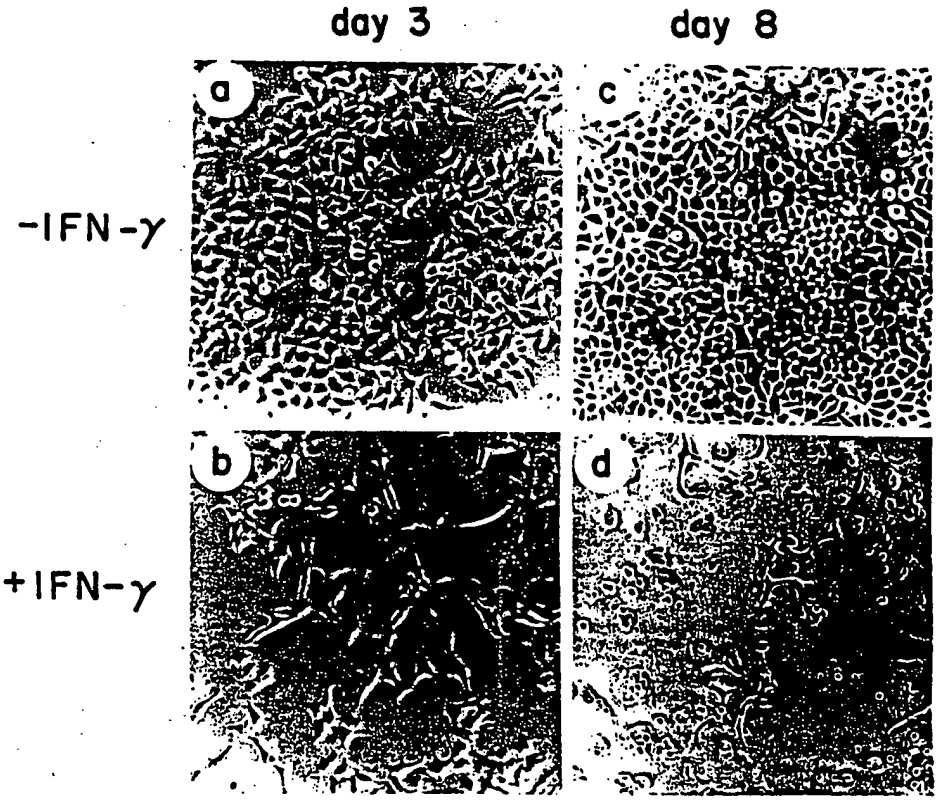

FIG. 3A(b) FIG. 3A(d)

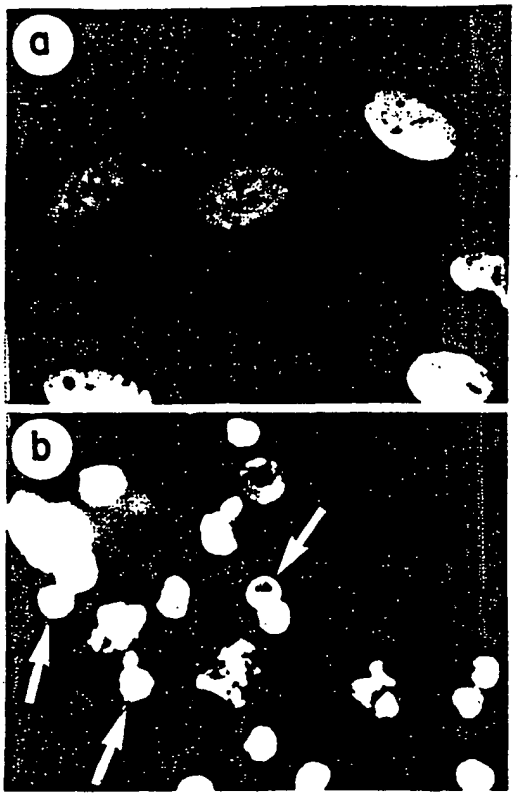

FIG. 3B(a)

FIG. 3B(b)

FIG. 3C(a) FIG. 3C(c) FIG. 3C(e)

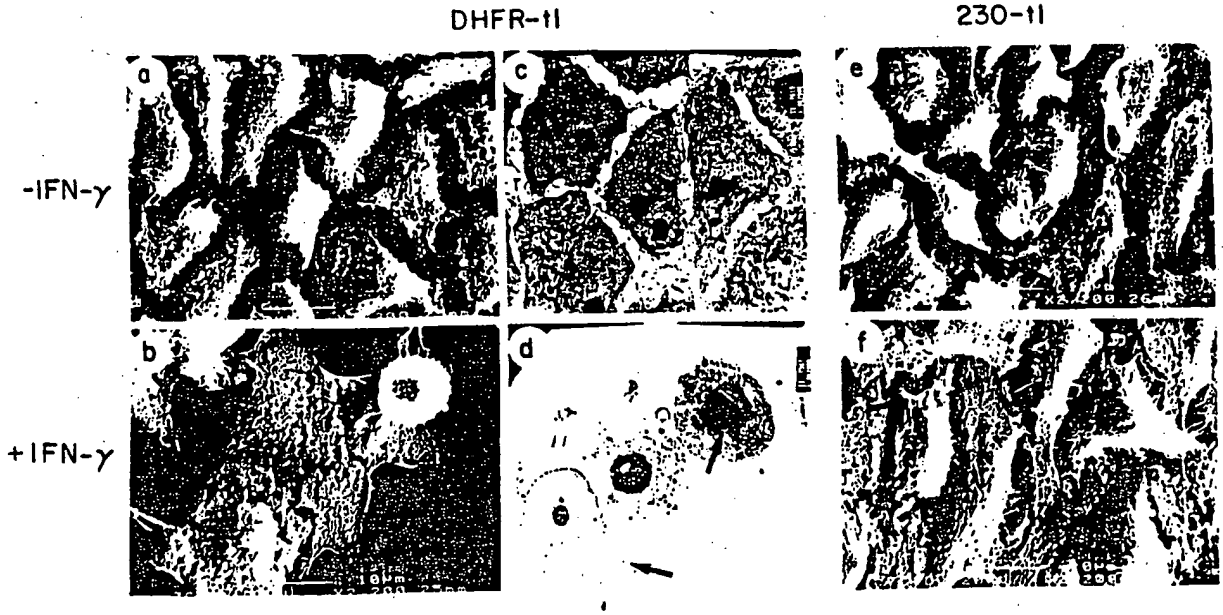

FIG. 3C(b) FIG. 3C(d) FIG. 3C(f)

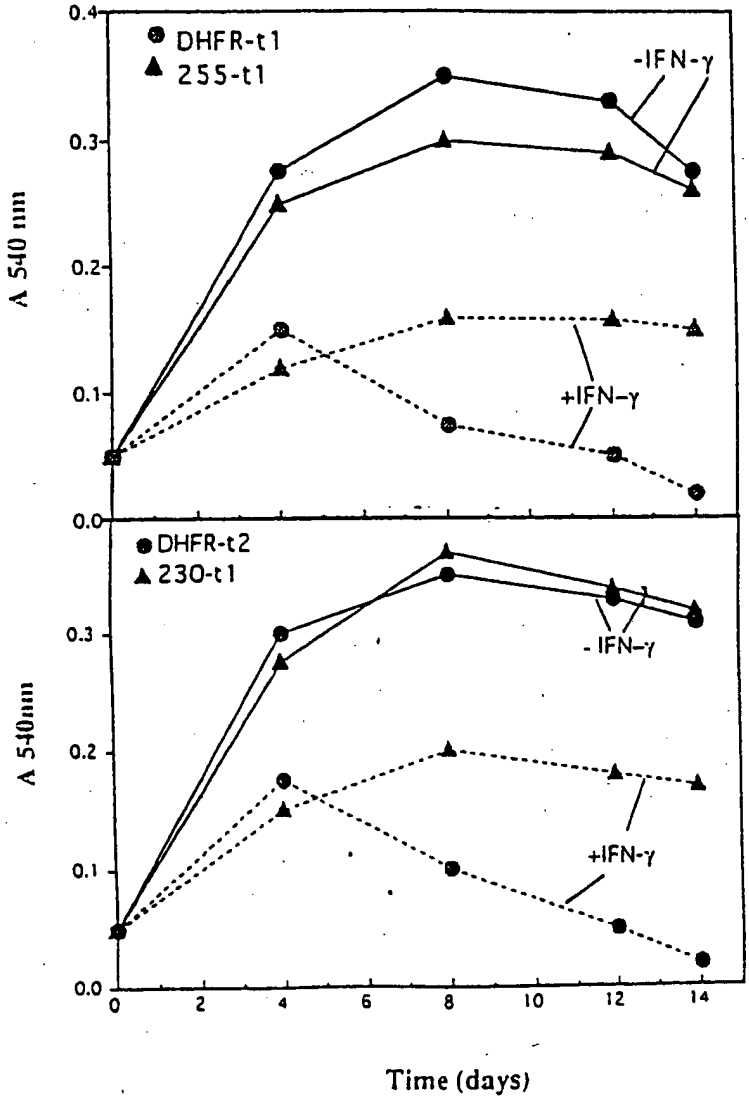

FIG. 4A

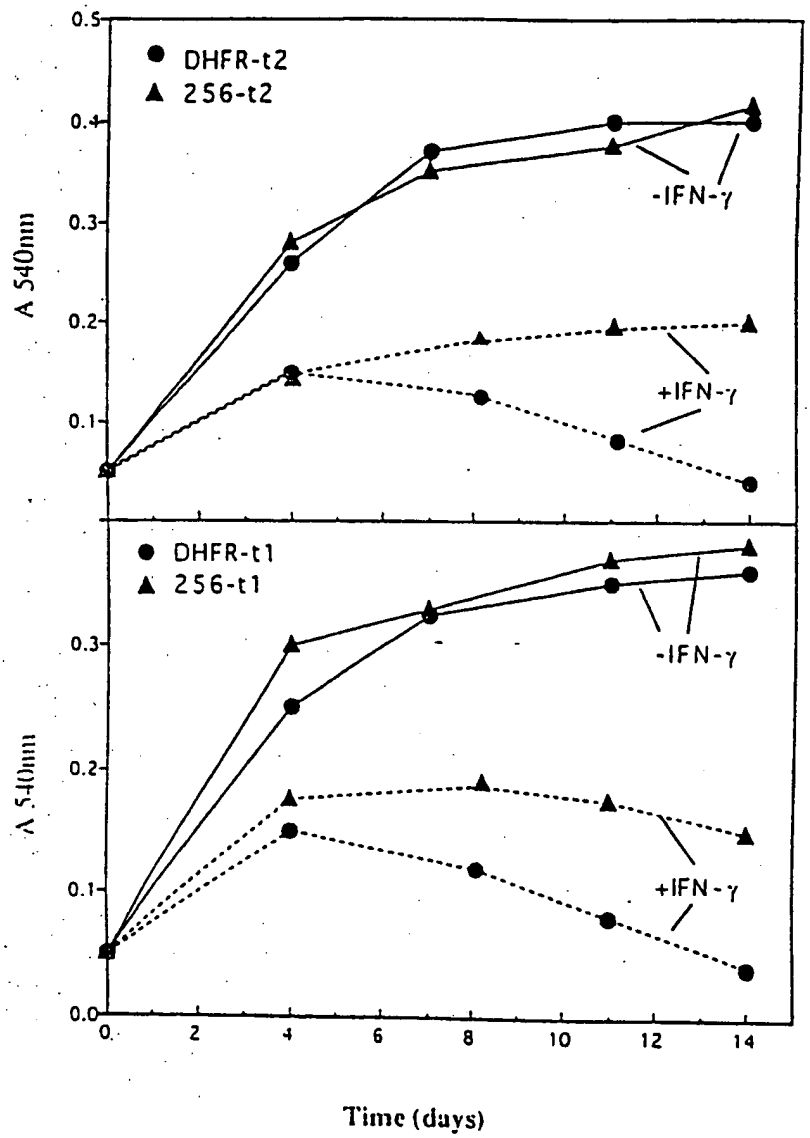

FIG. 4B

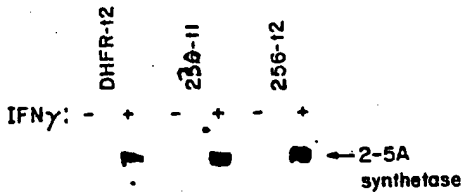

FIG. 4C

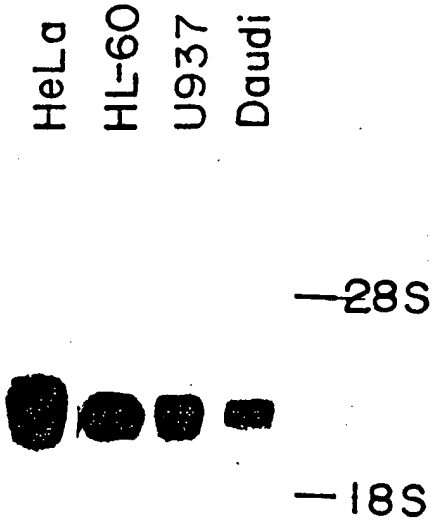

FIG. 10

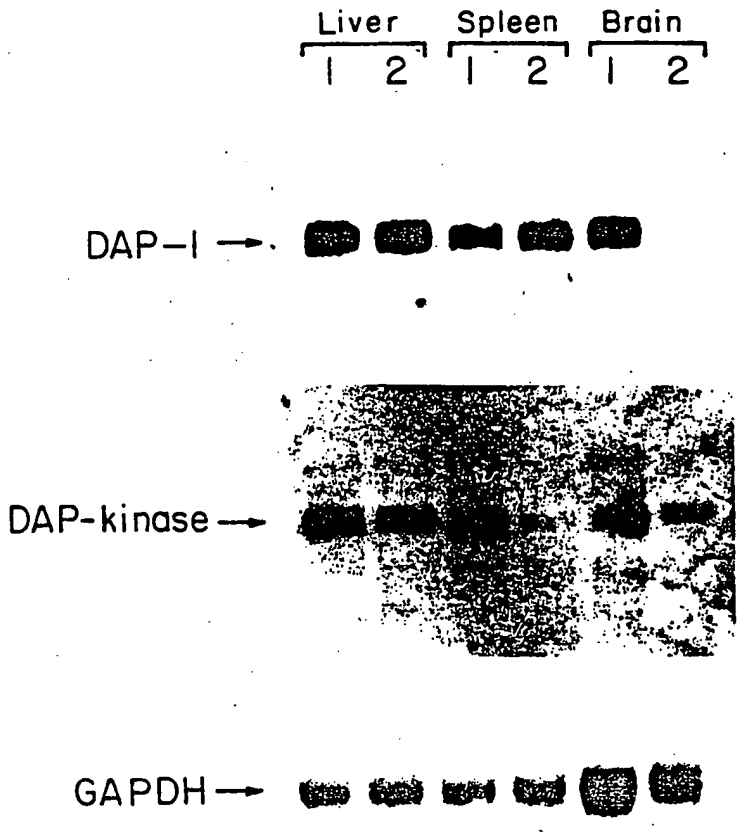

FIG. 11

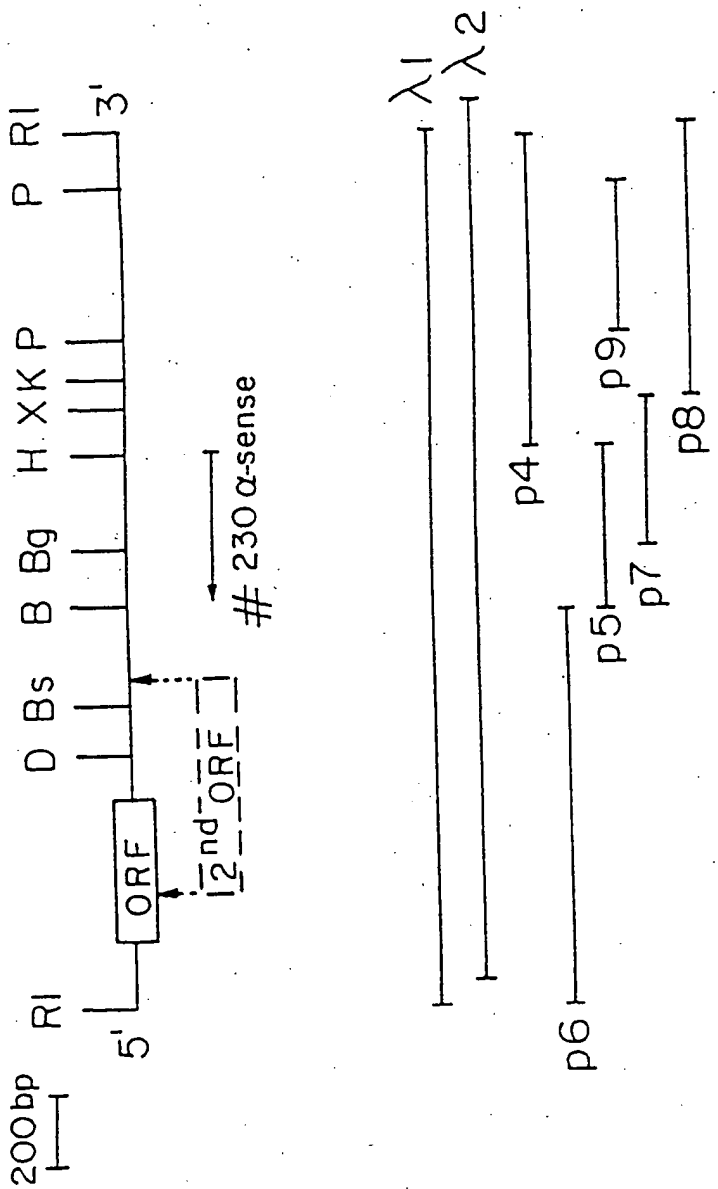

FIG. 5

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CGTGGCACTCACCCGGCTCGCGCGGCCCCGGCCGCCCACGCCGCGCGTCGTTCTCCCGCCCGCTCGCTCCCCGGCGCTCACACCTGAGCT      90

                                                                  M  S  S  P  P  E  G        7
CACTCGCGCACGCCCGCCCGGCCCGAGAACCGCGCCGCCGCCTCGGCCCCGCGGAAGCCCCGCCGCGCCATGTCTTCGCCTCCCGAAGGG    180

K  L  E  T  K  A  G  H  P  P  A  V  K  A  G  G  M  R  I  V  Q  K  H  P  H  T  G  D  T  K       37
AAACTAGAGACTAAAGCTGGACACCCGCCCGCCGTGAAAGCTGGTGGAATGCGAATTGTGCAGAAACACCCACATACAGGAGACACCAAA    270

E  E  K  D  K  D  D  Q  E  W  E  S  P  S  P  P  K  P  T  V  F  I  S  G  V  I  A  R  G  D       67
GAAGAGAAAGACAAGGATGACCAGGAATGGGAAAGCCCCAGTCCACCTAAACCCACTGTGTTCATCTCTGGGGTCATCGCCCGGGGTGAC    360

K  D  F  P  P  A  A  A  Q  V  A  H  Q  K  P  H  A  S  M  D  K  H  P  S  P  R  T  Q  H  I       97
AAAGATTTCCCCCCGGCGGCTGCGCAGGTGGCTCACCAGAAGCCGCATGCCTCCATGGACAAGCATCCTTCCCCAAGAACCCAGCACATC    450

                                                                                              102
Q  Q  P  R  K  *
CAGCAGCCACGCAAGTGAGCCTGGAGTCCACCAGCCTGCCCCATGGCCCCGGCTCTGCTGCACTTGGTATTTCCCTGACAGAGAGAACCA    540
GCAGTTTCGCCCAAATCCTACTCTGCTGGGAAATCTAAGGCAAAACGAAGTGCTCTGTCCTTTGCCTTACATTTCCATATTTAAAACTAG    630
AAACAGCTTCAGCCCAAACCTTGTTTATGGGGAGTCTGGTTGGATGTCATTTGAGGATCATTGTGCCCCTAGAGGTGCCATTAGCAGAAT    720
TTGCCAAGATCCGAGAAAAATTTTAGCTTTAGTTCTATTTCAGCAGTCACCTGACGTCCTTGTCTATGGTCTTAAAAACAAGAAGGCACA    810
CATTTGAGAAGATGAGATTAAGGTTAGGAGAAAACCTCAGTCATTGCATGCTTTTTAGTATGGGCCAATAAAATCTCAACACCTGTGGGA    900
GAGTAAGAACTAAGGGAATGAGTTTGGGCGCCCCCTCATAAAGGACCTTAGAGGCAGGGAACAGCAATGCCAAATTTCCCTCTCTCGTGA    990
GATGGGGGATCCTGTGCAGGCTGATGAGGCACCCATGAGAAAAGCCGAAAAAGCATGCATCTTAGAAATAGCCCCTCAATTCCAGGAGTC   1080
AACATGCCAAAGAATGAGGCTGGAGACAGGTAGCTCCGAGGGAGGACTTCTGGCATGAGATCTCGGCACGGCAAGCCCAGCATCGCCTCA   1170
GCCCAGACAGGCTCCACCAGGAGATCAAGCAAGGGCTGCCTTTCAGGAGTCACCTCCTGAGCCACTTCAGAGTTCTGGAAGTGACCACGG   1260
ACCAGGGTGGAGGAATAGACTTCTAGTTCATTCTGGGACACTTGAGCCAGAGAGTTGAAAGCTTGGAAAGACCAGATAAGAAACCTGCCC   1350
TTTGTCTCCCTAGGGACATGAGACACCACATTCCATTTGTGCTAGAAAAACCTATCCACTGATGAGTCTAACTGTTCCAAACGCCTCCCA   1440
CCTGGTGTGCACAGCTGCCTGGGTCCATTGTCACTTGGGTGCATCAGGTTGTCCTCCGATTTTTAGATGAGTTTCCTGTCTAGAGATGTC   1530
CTAGTCTGCTCACTGGCTGGTGGCAGTAGGGTACCCTGCGTCCTCGAAAAGCCAGAGGGTTCACCTAGTCAGACGAAACTCCAGAACAGT   1620
GCTTGTGGAGGGCCTGACTGTCCTGCTCACCCACAGCCGATCTGCTGCAGGTCAGCAACTGTGTCGTGAGCAGCTGCCAACCACCAGCCT   1710
TTCTGGTGCTGTTCTCCAGTTCACGTCTGCCAGCTGGTGAGGGCAGAGGCAGACCTGGTCAGACCCAGCGCCCCTCCTCCCTGAGGGAGC   1800
ATGGCACAGCCTCACACTTGAAAGACGGTGTTTGGTTTCCCATCTAATCAACTTAAGGGAAGCCGGCATGTACCCTTCAAGGCCCTGTCA   1890
CCACCTATTTTCCTGATCAGTTGGTATAAACTGAGGGTGGCTTTTAGAGACCCAGACTTGGTTGGCAGCGCTGCCATGGAACACCCCAGC   1980
AAGCACCTCCCAGCCTGCCTTTCGGAGCAGCACCCAGGAGGGGATGCCGCGCTCCAGCAACACCAGGTCAGGCCTGTGCAGACCCCTGCC   2070
CTGCCGCTGCAGAAATCCAGAAGCATCCTTAATGCTTCTCAGTCTTCAGCCAGAGGGAGGGCTGTTATTTCCAGAGGTGCGCTTTTTATG   2160
TACTTTTAGCTAGATGTGGCATGCATCTGTGAGCTTTAGATCATTAAATCCAAAATGTTTGCCTAAATGAGG                      2232
```

FIG. 6

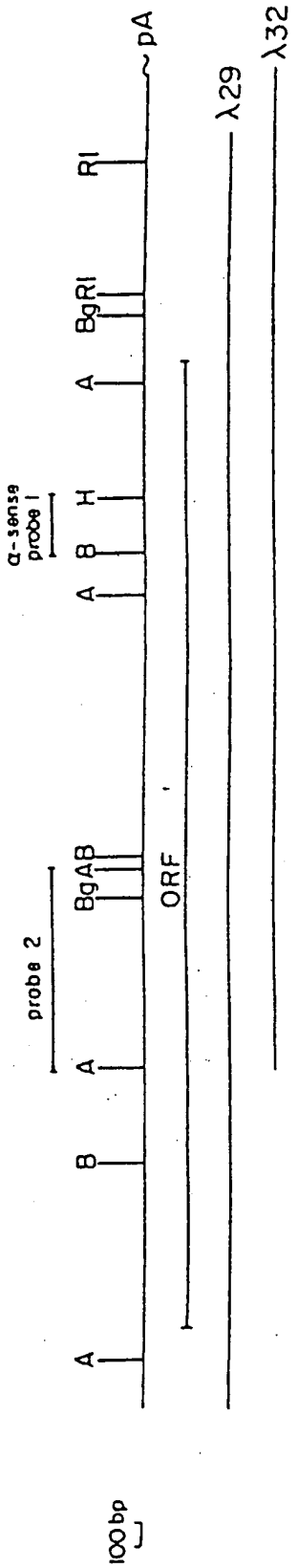


FIG. 7

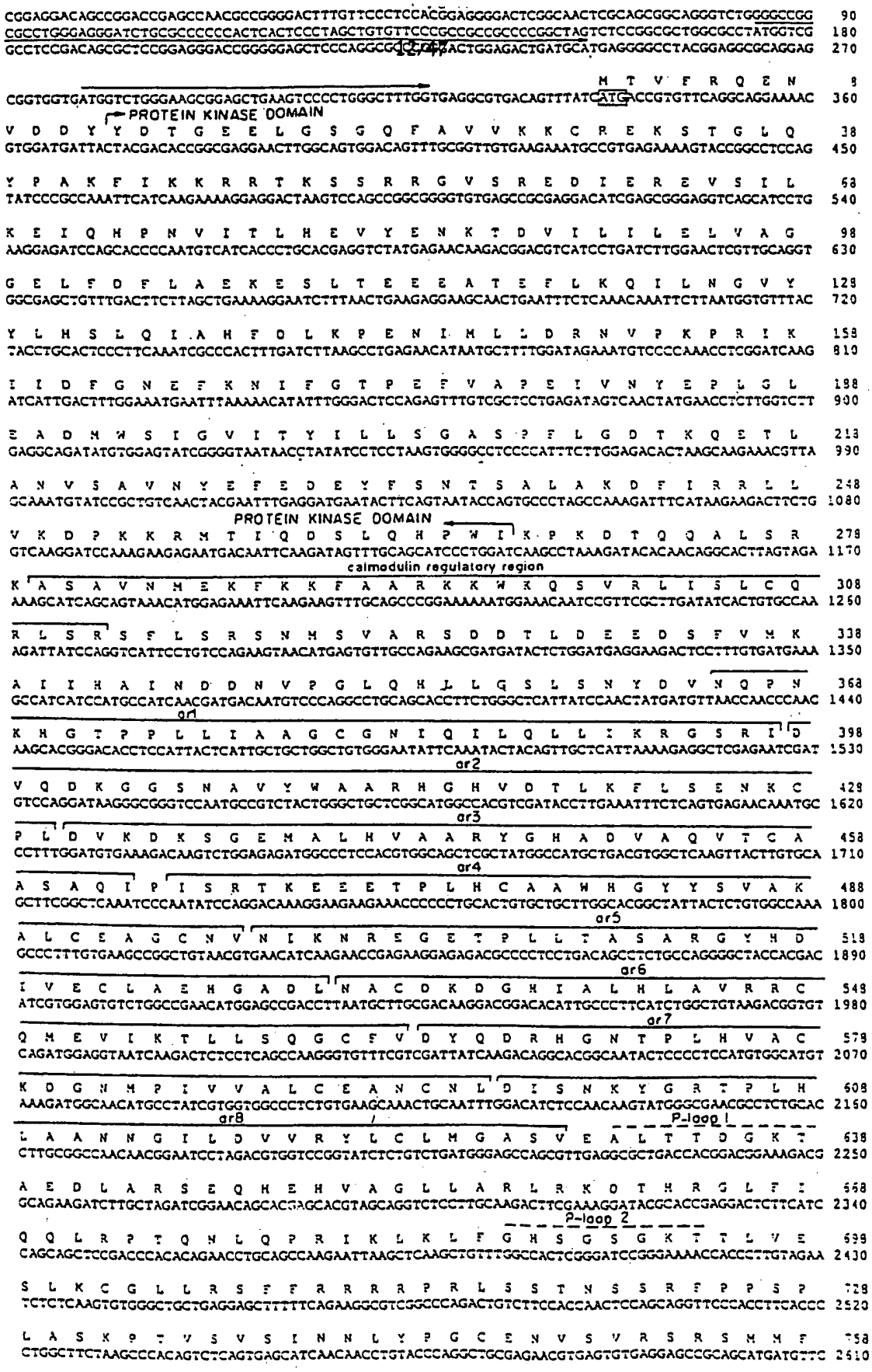


FIG. 8

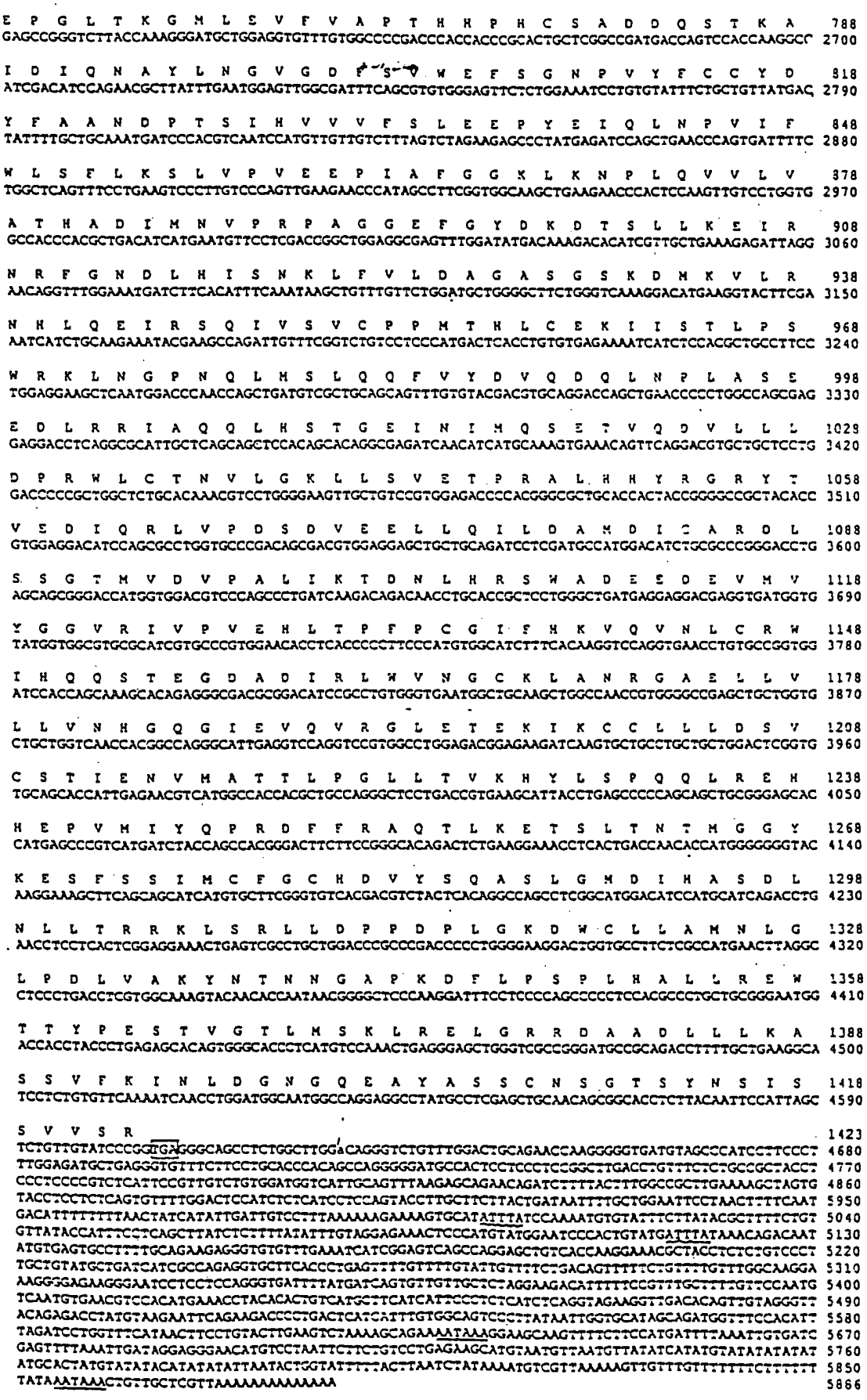


FIG. 8 (cont.)

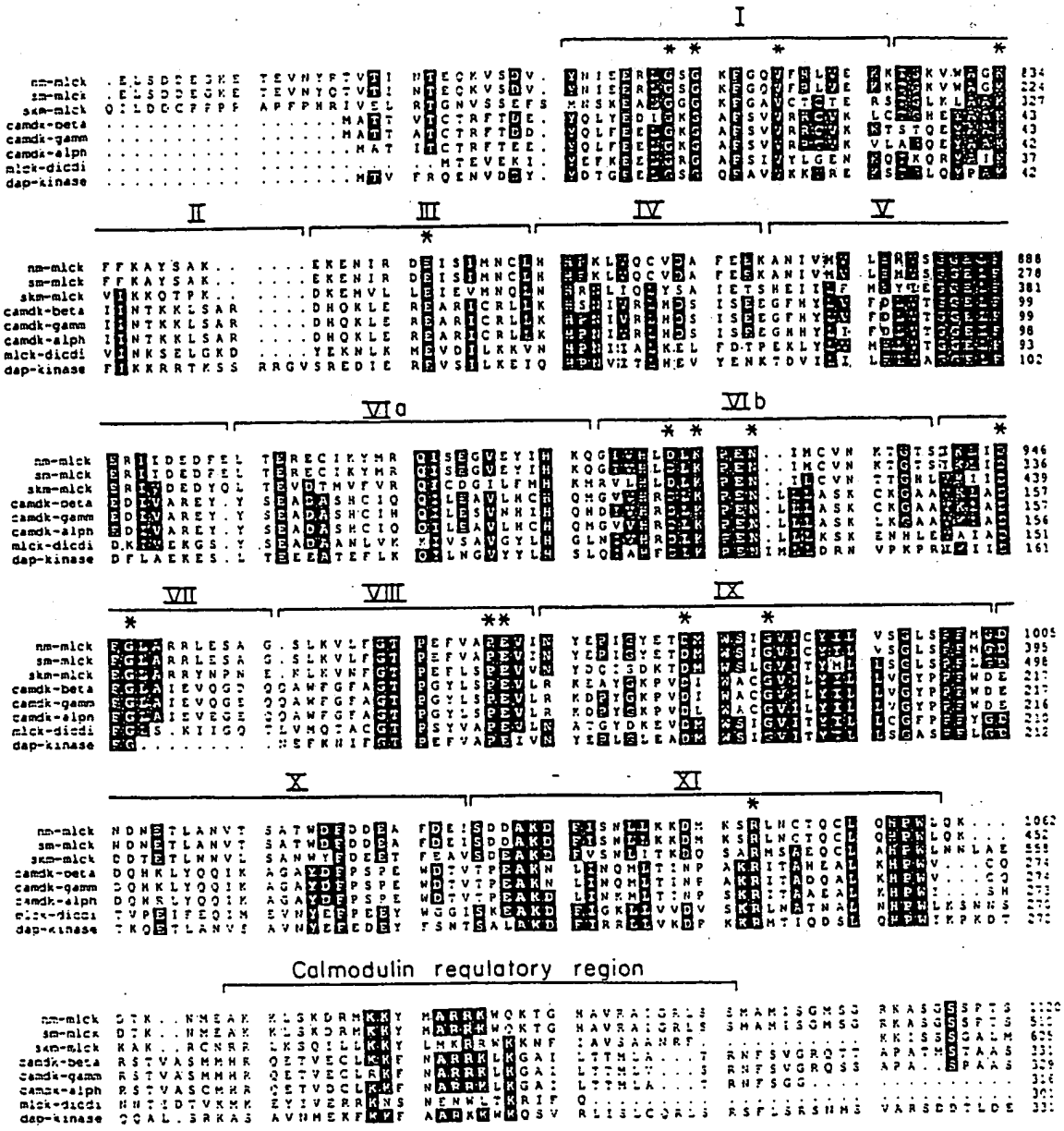


FIG. 9A

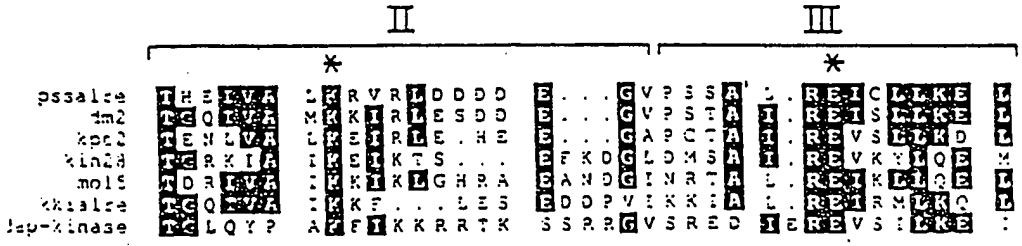


FIG. 9B

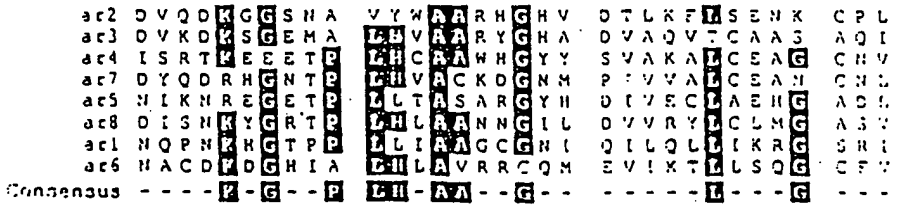


FIG. 9C

```
   1 GAATTCCGCCGGCCCCAGGCAGCGTGTGTCGGTCGCCTAGGCTGGAGAACTAGTCCTCGA   60
  61 CTCACGTGCAAGGATGATGCTGAAAGGAATAACAAGGCTTATCTCTAGGATCCATAAGTT  120
                     M  M  L  K  G  I  T  R  L  I  S  R  I  H  K  L
 121 GGACCCTGGGCGTTTTTTACACATGGGGACCCAGGCTCGCCAAAGCATTGCTGCTCACCT  180
       D  P  G  R  F  L  H  M  G  T  Q  A  R  Q  S  I  A  A  H  L
 181 AGATAACCAGGTTCCAGTTGAGAGTCCGAGAGCTATTTCCCGCACCAATGAGAATGACCC  240
       D  N  Q  V  P  V  E  S  P  R  A  I  S  R  T  N  E  N  D  P
 241 GGCCAAGCATGGGGATCAGCACGAGGGTCAGCACTACAACATCTCCCCCCAGGATTTGGA  300
       A  K  H  G  D  Q  H  E  G  Q  H  Y  N  I  S  P  Q  D  L  E
 301 GACTGTATTTCCCCATGGCCTTCCTCCTCGCTTTGTGATGCAGGTGAAGACATTCAGTGA  360
       T  V  F  P  H  G  L  P  P  R  F  V  M  Q  V  K  T  F  S  E
 361 AGCTTGCCTGATGGTAAGGAAACCAGCCCTAGAACTTCTGCATTACCTGAAAAACACCAG  420
       A  C  L  M  V  R  K  P  A  L  E  L  L  H  Y  L  K  N  T  S
 421 TTTTGCTTATCCAGCTATACGATATCTTCTGTATGGAGAGAAGGGAACAGGAAAAACCCT  480
       F  A  Y  P  A  I  R  Y  L  L  Y  G  E  K  G  T  G  K  T  L
 481 AAGTCTTTGCCATGTTATTCATTTCTGTGCAAAACAGGACTGGCTGATACTACATATTCC  540
       S  L  C  H  V  I  H  F  C  A  K  Q  D  W  L  I  L  H  I  P
 541 AGATGCTCATCTTTGGGTGAAAAATTGTCGGGATCTTCTGCAGTCCAGCTACAACAAACA  600
       D  A  H  L  W  V  K  N  C  R  D  L  L  Q  S  S  Y  N  K  Q
 601 GCGCTTTGATCAACCTTTAGAGGCTTCAACCTGGCTGAAGAATTTCAAAACTACAAATGA  660
       R  F  D  Q  P  L  E  A  S  T  W  L  K  N  F  K  T  T  N  E
 661 GCGCTTCCTGAACCAGATAAAAGTTCAAGAGAAGTATGTCTGGAATAAGAGAGAAAGCAC  720
       R  F  L  N  Q  I  K  V  Q  E  K  Y  V  W  N  K  R  E  S  T
 721 TGAGAAAGGGAGTCCTCTGGGAGAAGTGGTTGAACAGGGCATAACACGGGTGAGGAACGC  780
       E  K  G  S  P  L  G  E  V  V  E  Q  G  I  T  R  V  R  N  A
 781 CACAGATGCAGTTGGAATTGTGCTGAAAGAGCTAAAGAGGCAAAGTTCTTTGGGTATGTT  840
       T  D  A  V  G  I  V  L  K  E  L  K  R  Q  S  S  L  G  M  F
 841 TCACCTCCTAGTGGCCGTGGATGGAATCAATGCTCTTTGGGGAAGAACCACTCTGAAAAG  900
       H  L  L  V  A  V  D  G  I  N  A  L  W  G  R  T  T  L  K  R
 901 AGAAGATAAAAGCCCGATTGCCCCCGAGGAATTAGCACTTGTTCACAACTTGAGGAAAAT  960
       E  D  K  S  P  I  A  P  E  E  L  A  L  V  H  N  L  R  K  M
 961 GATGAAAAATGATTGGCATGGAGGCGCCATTGTGTCGGCTTTGAGCCAGACTGGGTCTCT 1020
       M  K  N  D  W  H  G  G  A  I  V  S  A  L  S  Q  T  G  S  L
1021 CTTTAAGCCCCGGAAAGCCTATCTGCCCCAGGAGTTGCTGGGAAAGGAAGGATTTGATGC 1080
       F  K  P  R  K  A  Y  L  P  Q  E  L  L  G  K  E  G  F  D  A
1081 CCTGGATCCCTTTATTCCCATCCTGGTTTCCAACTATAACCCAAAGGAATTTGAAAGTTG 1140
       L  D  P  F  I  P  I  L  V  S  N  Y  N  P  K  E  F  E  S  C
1141 TATTCAGTATTATTTGGAAAACAATTGGCTTCAACATGAGAAAGCTCCTACAGAAGAAGG 1200
       I  Q  Y  Y  L  E  N  N  W  L  Q  H  E  K  A  P  T  E  E  G
1201 GAAAAAAGAGCTGCTGTTCCTAAGTAACGCGAACCCCTCGCTGCTGGAGCGGCACTGTGC 1260
       K  K  E  L  L  F  L  S  N  A  N  P  S  L  L  E  R  H  C  A
1261 CTACCTCTAAGCCAAGATCACAGCATGTGAGGAAGACAGTGGACATCTGCTTTATGCTGG 1320
       Y  L
1321 ACCCAGTAAGATGAGGAAGTCGGGCAGTACACAGGAAGAGGAGCCAGGCCCTTGTACCTA 1380
1381 TGGGATTGGACAGGACTGCAGTTGGCTCTGGACCTGCATTAAAATGGGTTTCACTGTGAA 1440
1441 TGCGTGACAATAAGATATTCCCTTGTTCCTAAAACTTTATATCAGTTTATTGGATGTGGG 1500
1501 TTTtTCACATTTAAGATAATTATGGCTCTTTTCCTAAAAAATAAAATATCTTTCTAAAAA 1560
1561 AAAAAAAA 1568
```

FIG. 12

CTAGATGAGGCAGATATAAGAGTCA 25

TGGAAAAAAGGACAGAGAAAAAAA 50

CAGACAAATCAGTTGTCAGTATCCA 75

TGGCCTCTGATTCTGTCTCAACCAT 100

GAAACAGAAGTGACACATATAC 122

CTGCTAAAAG

FIG. 13

```
                                                      M  Q  P
  1 GGCTATAAGCGCACGGCCTCGGCGACCCTCTCCGACCCGGCCGCCGCCGCCATGCAGCCC   60
    S  S  L  L  P  L  A  L  C  D  E  A  A  P  A  S  A  L  V  R
 61 TCCAGCCTTCTGCCGCTCGCCCTCTGCCTGCTGGCTGCACCCGCCTCCGCGCTCGTCAGG  120
    I  P  L  H  K  F  T  S  I  R  R  T  M  S  E  V  G  G  S  V
121 ATCCCGCTGCACAAGTTCACGTCCATCCGCCGGACCATGTCGGAGGTTGGGGGCTCTGTG  180
    E  D  L  I  A  K  G  P  V  S  K  Y  S  Q  A  V  P  A  V  T
181 GAGGACCTGATTGCCAAAGGCCCCGTCTCAAAGTACTCCCAGGCGGTGCCAGCCGTGACC  240
    E  G  P  I  P  E  V  L  K  N  Y  M  D  A  Q  Y  Y  G  E  I
241 GAGGGGCCCATTCCCGAGGTGCTCAAGAACTACATGGACGCCCAGTACTACGGGGAGATT  300
    G  I  G  T  P  P  Q  C  F  T  V  V  F  D  T  G  S  S  N  L
301 GGCATCGGGACGCCCCCCCAGTGCTTCACAGTCGTCTTCGACACGGGCTCCTCCAACCTG  360
    W  V  P  S  I  H  C  K  L  L  D  I  A  C  W  I  H  H  K  Y
361 TGGGTCCCCTCCATCCACTGCAAACTGCTGGACATCGCTTGCTGGATCCACCACAAGTAC  420
    N  S  D  K  S  S  T  Y  V  K  N  G  T  S  F  D  I  H  Y  G
421 AACAGCGACAAGTCCAGCACCTACGTGAAGAATGGTACCTCGTTTGACATCCACTATGGC  480
    S  G  S  L  S  G  Y  L  S  Q  D  T  V  S  V  P  C  Q  S  A
481 TCGGGCAGCCTCTCCGGGTACCTGAGCCAGGACACTGTGTCGGTGCCCTGCCAGTCAGCG  540
    S  S  A  S  A  L  G  G  V  K  V  E  R  Q  V  F  G  E  A  T
541 TCGTCAGCCTCTGCCCTGGGCGGTGTCAAAGTGGAGAGGCAGGTCTTTGGGGAGGCCACC  600
    K  Q  P  G  I  T  F  I  A  A  K  F  D  G  I  L  G  M  A  Y
601 AAGCAGCCAGGCATCACCTTCATCGCAGCCAAGTTCGATGGCATCCTGGGCATGGCCTAC  660
    P  R  I  S  V  N  N  V  L  P  V  F  D  N  L  M  Q  Q  K  L
661 CCCCGCATCTCCGTCAACAACGTGCTGCCCGTCTTCGACAACCTGATGCAGCAGAAGCTG  720
    V  D  Q  N  I  F  S  F  Y  L  S  R  D  P  D  A  Q  P  G  G
721 GTGGACCAGAACATCTTCTCCTTCTACCTGAGCAGGGACCCAGATGCGCAGCCTGGGGGT  780
    E  L  M  L  G  G  T  D  S  K  Y  Y  K  G  S  L  S  Y  L  N
781 GAGCTGATGCTGGGTGGCACAGACTCCAAGTATTACAAGGGTTCTCTGTCCTACCTGAAT  840
    V  T  R  K  A  Y  W  Q  V  H  L  D  Q  V  E  V  A  S  G  L
841 GTCACCCGCAAGGCCTACTGGCAGGTCCACCTGGACCAGGTGGAGGTGGCCAGCGGGCTG  900
    T  L  C  K  E  G  C  E  A  I  V  D  T  G  T  S  L  M  V  G
901 ACCCTGTGCAAGGAGGGCTGTGAGGCCATTGTGGACACAGGCACTTCCCTCATGGTGGGC  960
    P  V  D  E  V  R  E  L  Q  K  A  I  G  A  V  P  L  I  Q  G
961 CCGGTGGATGAGGTGCGCGAGCTGCAGAAGGCCATCGGGGCCGTGCCGCTGATTCAGGGC 1020
    E  Y  M  I  P  C  E  K  V  S  T  L  P  A  I  T  L  K  L  G
1021 GAGTACATGATCCCCTGTGAGAAGGTGTCCACCCTGCCCGCGATCACACTGAAGCTGGGA 1080
    G  K  G  Y  K  L  S  P  E  D  Y  T  L  K  V  S  Q  A  G  K
1081 GGCAAAGGCTACAAGCTGTCCCCAGAGGACTACACGCTCAAGGTGTCGCAGGCCGGGAAG 1140
    T  L  C  L  S  G  F  M  G  M  D  I  P  P  P  S  G  P  L  W
1141 ACCCTCTGCCTGAGCGGCTTCATGGGCATGGACATCCCGCCACCCAGCGGGCCACTCTGG 1200
    I  L  G  D  V  F  I  G  R  Y  Y  T  V  F  D  R  D  N  N  R
1201 ATCCTGGGCGACGTCTTCATCGGCCGCTACTACACTGTGTTTGACCGTGACAACAACAGG 1260
    V  G  F  A  E  A  A  R  L  *
1261 GTGGGCTTCGCCGAGGCTGCCCGCCTCTAGTTCCCAAGGCGTCCGCGCGCCAGCACAGAA 1320
1321 ACAGAGGAGAGTCCCAGAGCAGGAGGCCCCTGGCCCAGCGGCCCCTCCCACACACCCCA 1380
1381 CACACTCGCCCGCCCACTGTCCTGGGCGCCCTGGAAGCCGGCGGCCCAAGCCCGACTTGC 1440
1441 TGTTTTGTTCTGTGGTTTTCCCCTCCCTGGGTTCAGAAATGCTGCCTGCCTGTCTGTCTC 1500
1501 TCCATCTGTTTGGTGGGGGTAGAGCTGATCCAGAGCACAGATCTGTTTCGTGCATTGGAA 1560
1561 GACCCCACCCAAGCTTGGCAGCCGAGCTCGTGTATCCTGGGGCTCCCTTCATCTCCAGGG 1620
1621 AGTCCCCTCCCCGGCCCTACCAGCGCCCGCTGGGCTGAGGGCCCCTCTGCCCAGCTTGGG 1680
1681 TCCTCCCGGGCCCTCCCTTGGAAACCTGCCCTGCCTGAGGGCCCCTCTGCCCAGCTTGGG 1740
1741 CCCAGCTGGGCTCTGCCACCCTACCTGTTCAGTGTCCCGGGCCCGTTGAGGATGAGGCCG 1800
1801 CTAGAGGCCTGAGGATGAGCTGGAAGGAGTGAGAGGGGACAAAACCCACCTTGTTGGAGC 1860
1861 CTGCAGGGTGGTGCTGGGACTGAGCCAGTCCCAGGGGCATGTATTGGCCTGGAGGTGGGG 1920
1921 TTGGGATTGGGGGCTGGTGCCAGCCTTCCTCTGCAGCTGACCTTGTTGTCCTCCCCTTG 1980
1981 GGCGGCTGAGAGCCCCAGCTGACATGGAAATACAGTTGTTGGCCTCCGGCCTCCCCTC 2038
```

FIG. 14

```
        10                                                  50
GAATTCCGCTCTATGGAGGTGGCAGCGGGTACCGAGTGGCGGCTGCAGCAGCGACTCCTC

        70                  90                  110
TGAGCTGAGTTTGAGGCCGTCCCCGACTCCTTCCTCCCCCTTCCCTCCCCCTTTTTTTTG

       130                 150                 170
TTTTCCGTTCCCCTTTCCCCTCCCTTCCCTATCCCCGACGACCGGATCCTGAGGAGGGCA

       190                 210                 230
GCTGCGGTGGCAGCTGCTGAGTTCTCGGTGAAGGTATTTCATTTCTCCTGTCCCCTCCCC
                      V   L   G   E   G   I   S   F   L   L   S   P   P   L

       250                 270                 290
TCCCCACCCCATCTATTAATATTATTCTTTTGAAGATTCTTCGTTGTCAAGCCGCCAAAG
  P   T   P   S   I   N   I   I   L   L   K   I   L   R   C   Q   A   A   K   V

       310                 330                 350
TGGAGAGTGCGATTGCAGAAGGGGGTGCTTCTCGTTTCAGTGCTTCTCGGGGCGGAGGAG
  E   S   A   I   A   E   G   G   A   S   R   F   S   A   S   S   G   G   G   G

       370                 390                 410
GAAGTAGGGGTGCACCTCAGCACTATCCCAAGACTGCTGGCAACAGCGAGTTCCTGGGGA
  S   R   G   A   P   Q   H   Y   P   K   T   A   G   N   S   E   F   L   G   K

       430                 450                 470
AAACCCCAGGGCAAAACGCTCAGAAATGGATTCCTGCACGAAGCACTAGACGAGATGACA
  T   P   G   Q   N   A   Q   K   W   I   P   A   R   S   T   R   R   D   D   N

       490                 510                 530
ACTCCGCAGCAAACAACTCCGCAAACGAAAAAGAACGACATGATGCAATCTTCAGGAAAG
  S   A   A   N   N   S   A   N   E   K   E   R   H   D   A   I   F   R   K   V

       550                 570                 590
TAAGAGGCATACTAAATAAGCTTACTCCTGAAAAGTTTGACAAGCTATGCCTTGAGCTCC
  R   G   I   L   N   K   L   T   P   E   K   F   D   K   L   C   L   E   L   L

       610                 630                 650
TCAATGTGGGTGTAGAGTCTAAACTCATCCTTAAAGGGGTCATACTGCTGATTGTGGACA
  N   V   G   V   E   S   K   L   I   L   K   G   V   I   L   L   I   V   D   K

       670                 690                 710
AAGCCCTAGAAGAGCCAAAGTATAGCTCACTGTATGCTCAGCTATGTCTGCGATTGGCAG
  A   L   E   E   P   K   Y   S   S   L   Y   A   Q   L   C   L   R   L   A   E

       730                 750                 770
AAGATGCACCAAACTTTGATGGCCCAGCAGCAGAGGGTCAACCAGGACAGAAGCAAAGCA
  D   A   P   N   F   D   G   P   A   A   E   G   Q   P   G   Q   K   Q   S   T

       790                 810                 830
CCACATTCAGACGCCTCCTAATTTCCAAATTACAAGATGAATTTGAAAACCGAACTAGAA
  T   F   R   R   L   L   I   S   K   L   Q   D   E   F   E   N   R   T   R   N

       850                 870                 890
ATGTTGATGTCTATGATAAGCGTGAAAATCCCCTCCTCCCCGAGGAGGAGGAACAGAGAG
  V   D   V   Y   D   K   R   E   N   P   L   L   P   E   E   E   E   Q   R   A
```

Fig. 15

```
      910                 930                 950
CCATTGCTAAGATCAAGATGTTGGGAAACTGCAAATTCATTGGAGAGCTTGGCAAGCTTG
  I  A  K  I  K  M  L  G  N  I  K  F  I  G  E  L  G  K  L  D

      970                 990                1010
ATCTTATTCACGAATCTATCCTTCATAAGTGCATCAAAACACTTTTGGAAAAGAAGAAGA
  L  I  H  E  S  I  L  H  K  C  I  K  T  L  L  E  K  K  K  R

     1030                1050                1070
GAGTCCAACTCAAAGATATGGGAGAGGATTTGGAGTGCCTCTGTCAGATAATGAGGACAG
  V  Q  L  K  D  M  G  E  D  L  E  C  L  C  Q  I  M  R  T  V

     1090                1110                1130
TGGGACCTAGATTAGACCATGAACGAGCCAAGTCCTTAATGGATCAGTACTTTGCCCGAA
  G  P  R  L  D  H  E  R  A  K  S  L  M  D  Q  Y  F  A  R  M

     1150                1170                1190
TGTGCTCCTTGATGTTAAGTAAGGAATTGCCAGCAAGGATTCGTTTCCTGCTGCAGGATA
  C  S  L  M  L  S  K  E  L  P  A  R  I  R  F  L  L  Q  D  T

     1210                1230                1250
CCGTAGAGTTGCGAGAACACCATTGGGTTCCTCGCAAGGCTTTTCTTGACAATGGACCAA
  V  E  L  R  E  H  H  W  V  P  R  K  A  F  L  D  N  G  P  K

     1270                1290                1310
AGACGATCAATCAAATTCGTCAAGATGCAGTAAAAGATCTAGGGGTGTTTATTCCTGCTC
  T  I  N  Q  I  R  Q  D  A  V  K  D  L  G  V  F  I  P  A  P

     1330                1350                1370
CTATGGCTCAAGGGATGAGAAGTGACTTCTTTCTGGAGGGACCGTTCATGCCACCCAGGA
  M  A  Q  G  M  R  S  D  F  F  L  E  G  P  F  M  P  P  R  M

     1390                1410                1430
TGAAAATGGATAGGGACCCACTTGGAGGACTTGCTGATATGTTTGGACAAATGCCAGGTA
  K  M  D  R  D  P  L  G  G  L  A  D  M  F  G  Q  M  P  G  S

     1450                1470                1490
GCGGAATTGGTACTGGTCCAGGAGTTATCCAGGATAGATTTTCACCCACCATGGGACGTC
  G  I  G  T  G  P  G  V  I  Q  D  R  F  S  P  T  M  G  R  H

     1510                1530                1550
ATCGTTCAAATCAACTCTTCAATGGCCATGGGGGACACATCATGCCTCCCACACAATCGC
  R  S  N  Q  L  F  N  G  H  G  G  H  I  M  P  P  T  Q  S  Q

     1570                1590                1610
AGTTTGGAGAGATGGGAGGCAAGTTTATGAAAAGCCAGGGGCTAAGCCAGCTCTACCATA
  F  G  E  M  G  G  K  F  M  K  S  Q  G  L  S  Q  L  Y  H  N

     1630                1650                1670
ACCAGAGTCAGGGACTCTTATCCCAGCTGCAAGGACAGTCGAAGGATATGCCACCTCGGT
  Q  S  Q  G  L  L  S  Q  L  Q  G  Q  S  K  D  M  P  P  R  F

     1690                1710                1730
TTTCTAAGAAAGGACAGCTTAATGCAGATGAGATTAGCCTGAGGCCTGCTCAGTCGTTCC
  S  K  K  G  Q  L  N  A  D  E  I  S  L  R  P  A  Q  S  F  L

     1750                1770                1790
```

Fig. 15 (con't)

```
TAATGAATAAAAATCAAGTGCCAAAGCTTCAGCCCCAGATAACTATGATTCCTCCTAGTG
 M  N  K  N  Q  V  P  K  L  Q  P  Q  I  T  M  I  P  P  S  A

      1810                1830                1850
CACAACCACCACGCACTCAAACACCACCTCTGGGACAGACACCTCAGCTTGGTCTCAAAA
 Q  P  P  R  T  Q  T  P  P  L  G  Q  T  P  Q  L  G  L  K  T

      1870                1890                1910
CTAATCCACCACTTATCCAGGAAAAGCCTGCCAAGACCAGCAAAAAGCCACCACCGTCAA
 N  P  P  L  I  Q  E  K  P  A  K  T  S  K  K  P  P  P  S  K

      1930                1950                1970
AGGAAGAACTCCTTAAACTAACTGAAACTGTTGTGACTGAATATCTAAATAGTGGAAATG
 E  E  L  L  K  L  T  E  T  V  V  T  E  Y  L  N  S  G  N  A

      1990                2010                2030
CAAATGAGGCTGTCAATGGTGTAAGAGAAATGAGGGCTCCTAAACACTTTCTTCCTGAGA
 N  E  A  V  N  G  V  R  E  M  R  A  P  K  H  F  L  P  E  M

      2050                2070                2090
TGTTAAGCAAAGTAATCATCCTGTCACTAGATAGAAGCGATGAAGATAAAGAAAAAGCAA
 L  S  K  V  I  I  L  S  L  D  R  S  D  E  D  K  E  K  A  S

      2110                2130                2150
GTTCTTTGATCAGTTTACTCAAACAGGAAGGGATAGCCACAAGTGACAACTTCATGCAGG
 S  L  I  S  L  L  K  Q  E  G  I  A  T  S  D  N  F  M  Q  A

      2170                2190                2210
CTTTCCTGAATGTATTGGACCAGTGTCCCAAACTGGAGGTTGACATCCCTTTGGTGAAAT
 F  L  N  V  L  D  Q  C  P  K  L  E  V  D  I  P  L  V  K  S

      2230                2250                2270
CCTATTTAGCACAGTTTGCAGCTCGTGCCATCATTTCAGAGCTGGTGAGCATTTCAGAAC
 Y  L  A  Q  F  A  A  R  A  I  I  S  E  L  V  S  I  S  E  L

      2290                2310                2330
TAGCTCAACCACTAGAAAGTGGCACCCATTTTCCTCTCTTCCTACTTTGTCTTCAGCAGT
 A  Q  P  L  E  S  G  T  H  F  P  L  F  L  L  C  L  Q  Q  L

      2350                2370                2390
TAGCTAAATTACAAGATCGAGAATGGTTAACAGAACTTTTTCAACAAAGCAAGGTCAATA
 A  K  L  Q  D  R  E  W  L  T  E  L  F  Q  Q  S  K  V  N  M

      2410                2430                2450
TGCAGAAAATGCTCCCAGAAATTGATCAGAATAAGGACCGCATGTTGGAGATTTTGGAAG
 Q  K  M  L  P  E  I  D  Q  N  K  D  R  M  L  E  I  L  E  G

      2470                2490                2510
GAAAGGGACTGAGTTTCTTATTCCCACTCCTCAAATTGGAGAAGGAACTGTTGAAGCAAA
 K  G  L  S  F  L  F  P  L  L  K  L  E  K  E  L  L  K  Q  I

      2530                2550                2570
TAAAGTTGGATCCATCCCCTCAAACCATATATAAATGGATTAAAGATAACATCTCTCCCA
 K  L  D  P  S  P  Q  T  I  Y  K  W  I  K  D  N  I  S  P  K

      2590                2610                2630
AACTTCATGTAGATAAAGGATTTGTGAACATCTTAATGACTAGCTTCTTACAGTACATTT
```

Fig. 15 (con't)

```
  L  H  V  D  K  G  F  V  N  I  L  M  T  S  F  L  Q  Y  I  S

      2650                                                2690
CTAGTGAAGTAAACCCCCCCAGCGATGAAACAGATTCATCCTCTGCTCCTTCCAAAGAAC
  S  E  V  N  P  P  S  D  E  T  D  S  S  S  A  P  S  K  E  Q

      2710                2730                2750
AGTTAGAGCAGGAAAAACAACTACTACTATCTTTCAAGCCAGTAATGCAGAAATTTCTTC
  L  E  Q  E  K  Q  L  L  L  S  F  K  P  V  M  Q  K  F  L  H

      2770                2790                2810
ATGATCACGTTGATCTACAAGTCAGTGCCCTGTATGCTCTCCAGGTGCACTGCTATAACA
  D  H  V  D  L  Q  V  S  A  L  Y  A  L  Q  V  H  C  Y  N  S

      2830                2850                2870
GCAACTTCCCAAAAGGCATGTTACTTCGCTTTTTTGTGCACTTCTATGACATGGAAATTA
  N  F  P  K  G  M  L  L  R  F  F  V  H  F  Y  D  M  E  I  I

      2890                2910                2930
TTGAAGAAGAAGCTTTCTTGGCTTGGAAAGAAGATATAACCCAAGAGTTTCCGGGAAAAG
  E  E  E  A  F  L  A  W  K  E  D  I  T  Q  E  F  P  G  K  G

      2950                2970                2990
GCAAGGCTTTGTTCCAGGTGAATCAGTGGCTAACCTGGTTAGAAACTGCTGAAGAAGAAG
  K  A  L  F  Q  V  N  Q  W  L  T  W  L  E  T  A  E  E  E  E

      3010                3030                3050
AATCAGAGGAAGAAGCTGACTAAAGAACCAGCCAAAGCCTTAAATTGTGCAAAACATACT
  S  E  E  E  A  D  ..

      3070                3090                3110
GTTGCTATGATGTAACTGCATTTGACCTAACCACTGCGAAAATTCATTCCGCTGTAATGT

      3130                3150                3170
TTTCACAATATTTAAAGCAGAAGCACGTCAGTTAGGATTTCCTTCTGCATAAGGTTTTTT

      3190                3210                3230
TGTAGTGTAATGTCTTAATCATAGTCTACCATCAAATATTTTAGGAGTATCTTTAATGTT

      3250                3270                3290
TAGATAGTATATTAGCAGCATGCAATAATTACATCATAAGTTCTCAAGCAGAGGCAGTCT

      3310                3330                3350
ATTGCAAGGACCTTCTTTGCTGCCAGTTATCATAGGCTGTTTTAAGCTAGAAAACTGAAT

      3370                3390                3410
AGCAACACTGAATACTGTAGAAATGCACTTTGCTCAGTAATACTTGAGTTGTTGCAATAT

      3430                3450                3470
TTGATTATCCATTTGGTTGTTACAGAAAAATTCTTAACTGTAATTGATGGTTGTTGCCGT

      3490                3510                3530
AATAGTATATTGCCTGTATTTCTACCTCTAGTAATGGGCTTTATGTGCTAGATTTTAATA

      3550                3570                3590
TCCTTGAGCCTGGGCAAGTGCACAAGTCTTTTTAAAAGAAACATGGTTTACTTGCACAAA

      3610                3630                3650
```

Fig. 15 (CON'T)

```
ACTGATCAGTTTTGaGAGATCGTTAATGCCCTTGAAGTGGTTTTTGTGGGTGTGAAACAA

      3670                3690                3710
ATGGTGAGAATTTGAATTGGTCCCTCCTATTATAGTATTGAAATTAAGTCTACTTAATTT

      3730                3750                3770
ATCAAGTCATGTTCATGCCCTGATTTTATATACTTGTATCTATCAATAAACATTGTGATA

      3790                3810
CTTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGGGAATTC
```

Fig. 15 (con't)

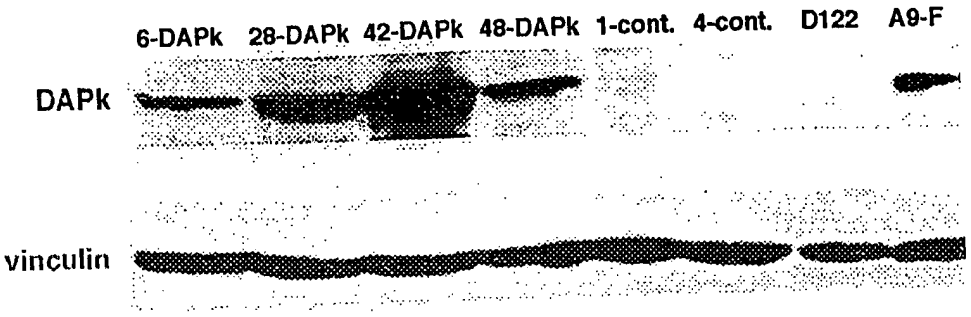


FIG. 16

FIG. 17

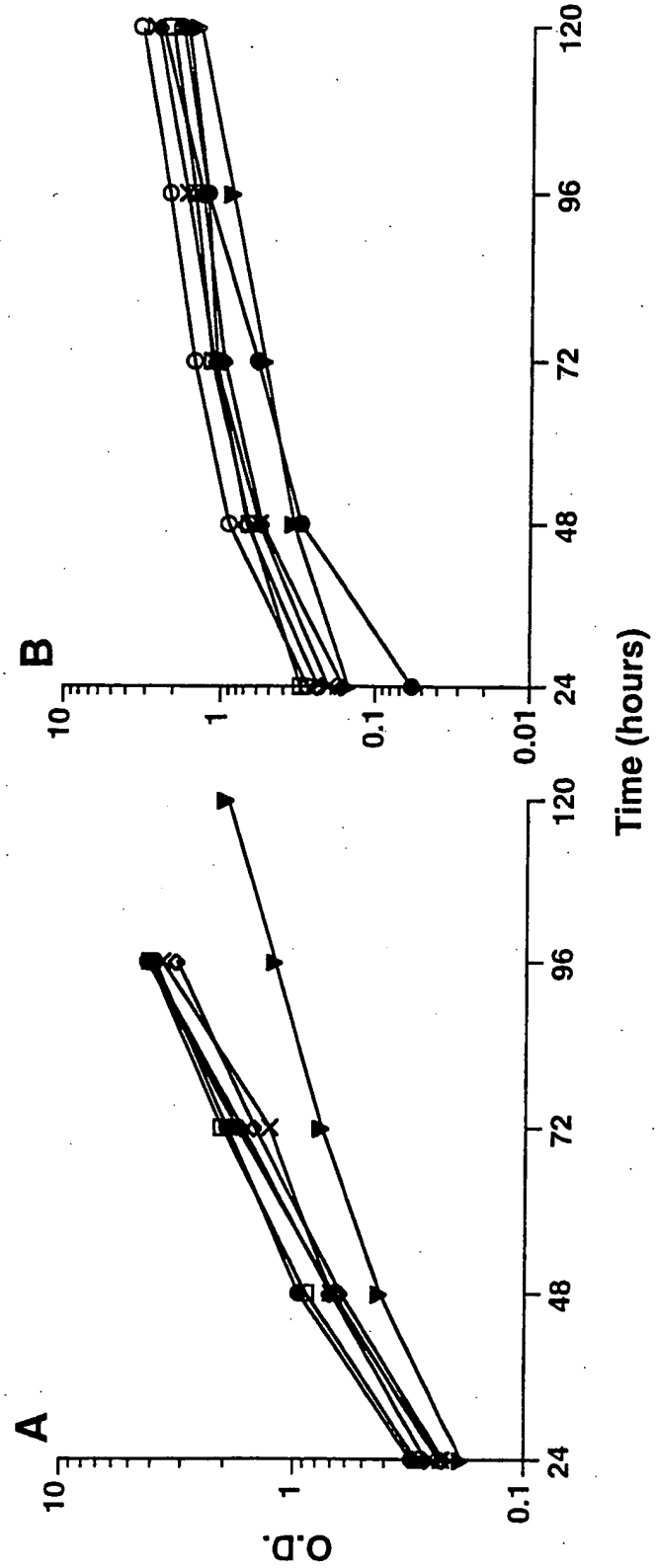


FIG. 18

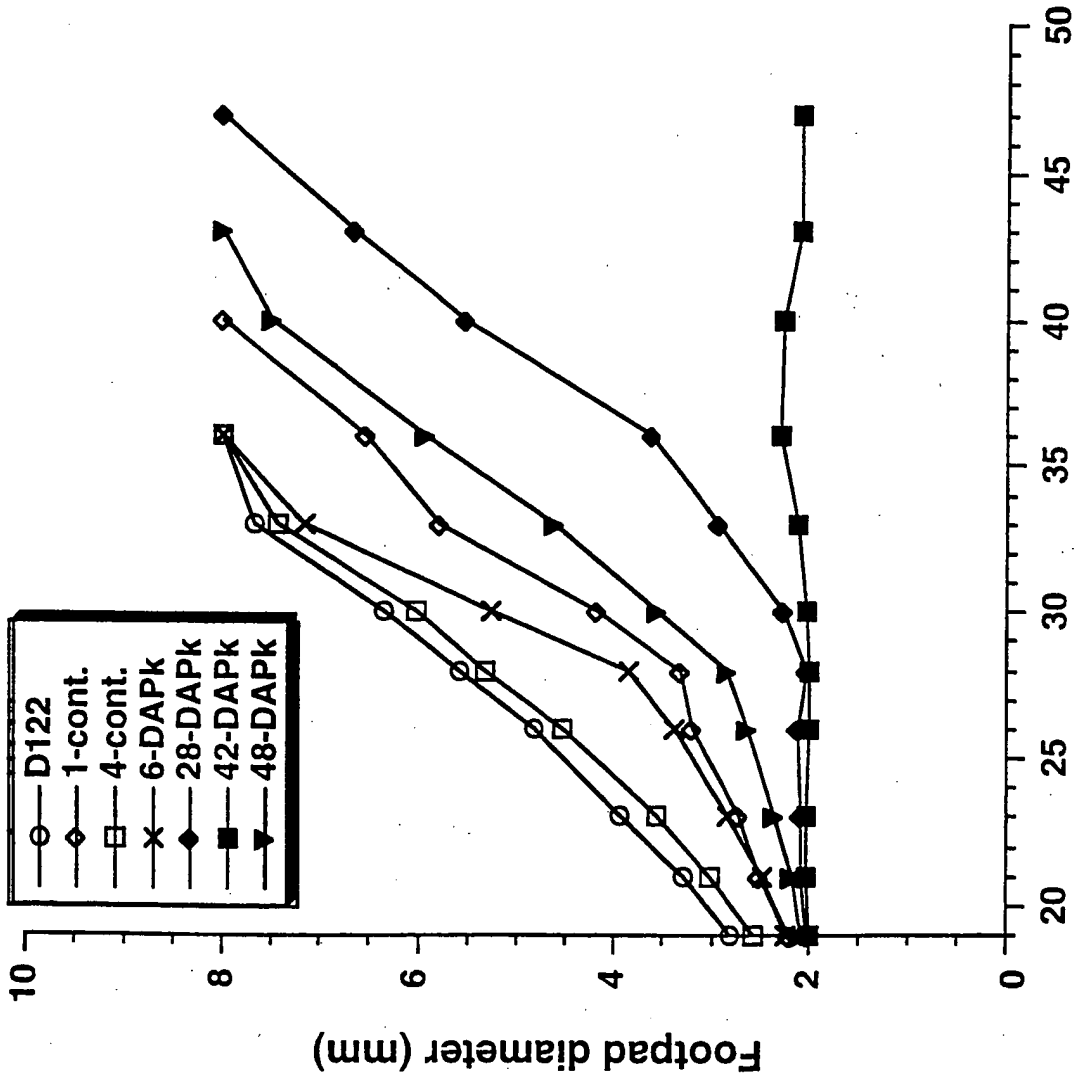
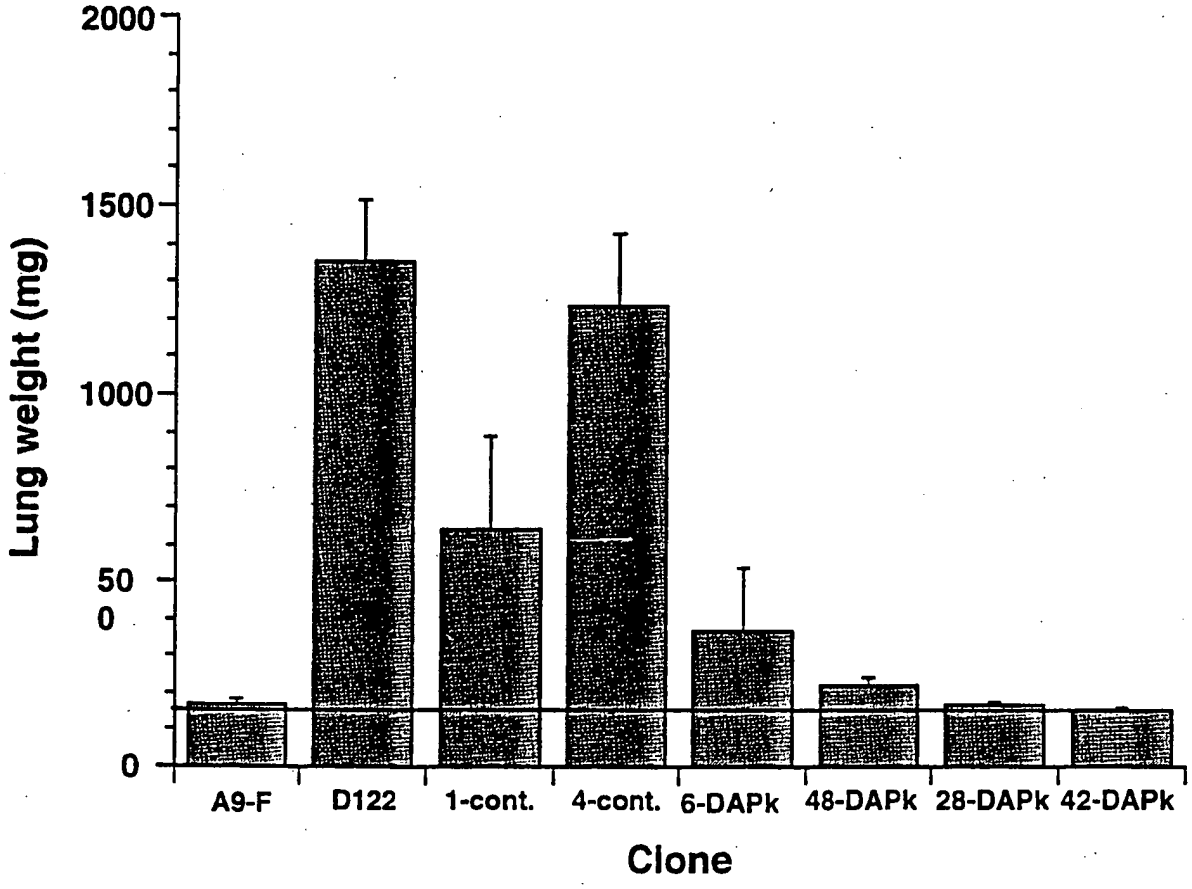


FIG. 19



clone	A9-F	D122	1-cont.	4-cont.	6-DAPk	48-DAPk	28-DAPk	42-DAPk
# of mice that developed lung metastasis	4/5	5/5	5/5	5/5	5/5	5/5	4/5	0/4
mean # of metastatic nodules per mouse	5±5	>100	>100	>100	34±27	12±7	3±2	0

FIG. 20

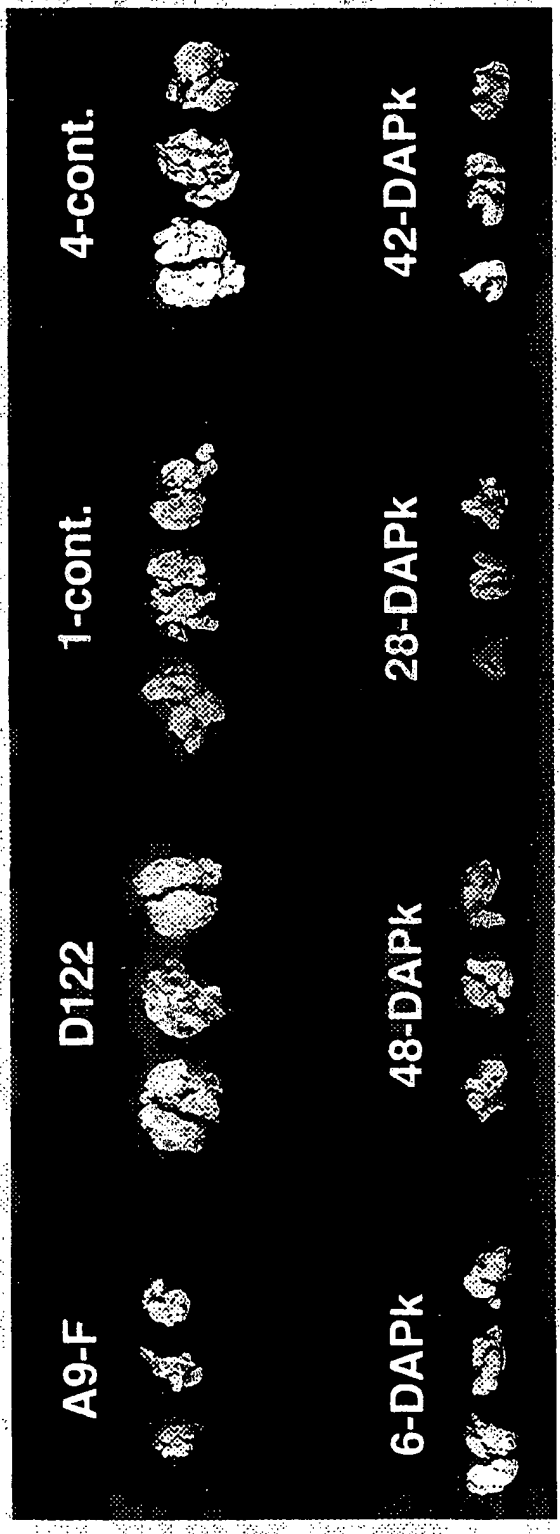


FIG. 21

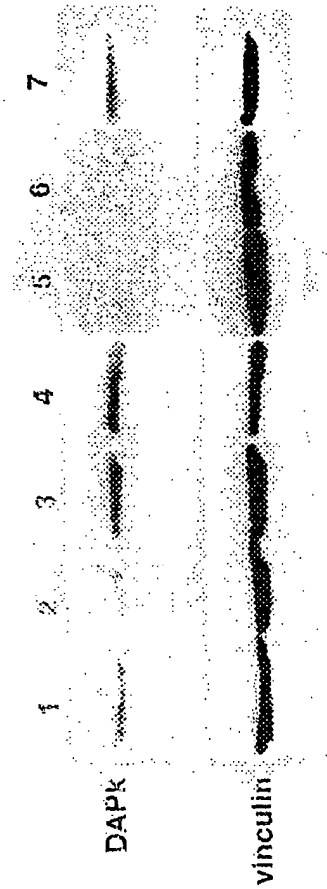


FIG. 22

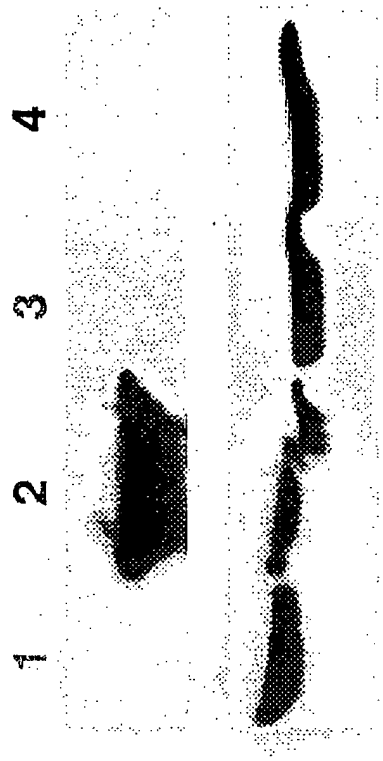


FIG. 24

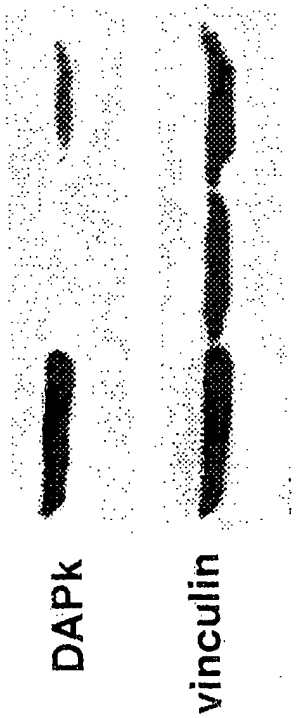


FIG. 23

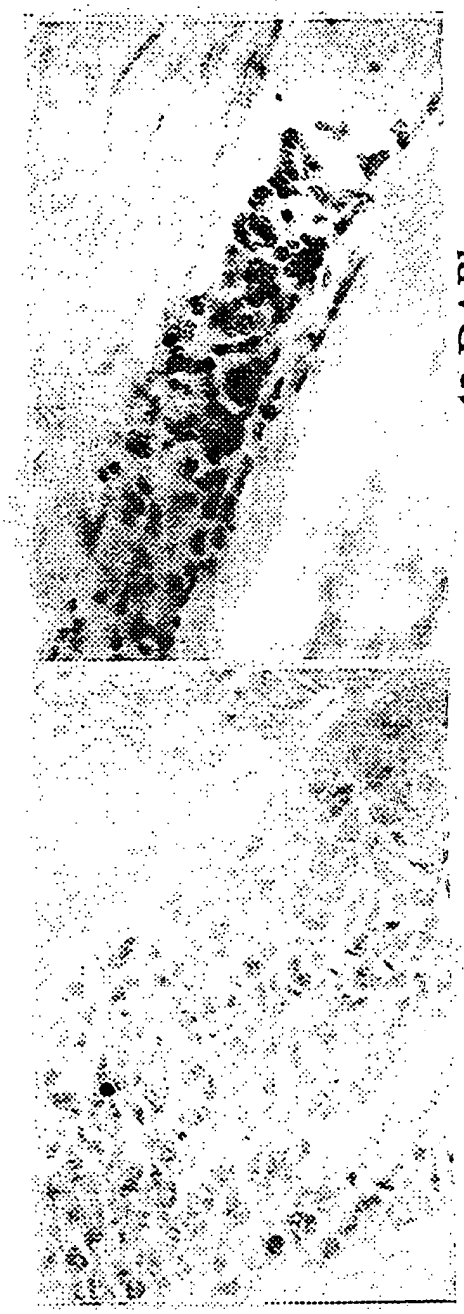


FIG. 25

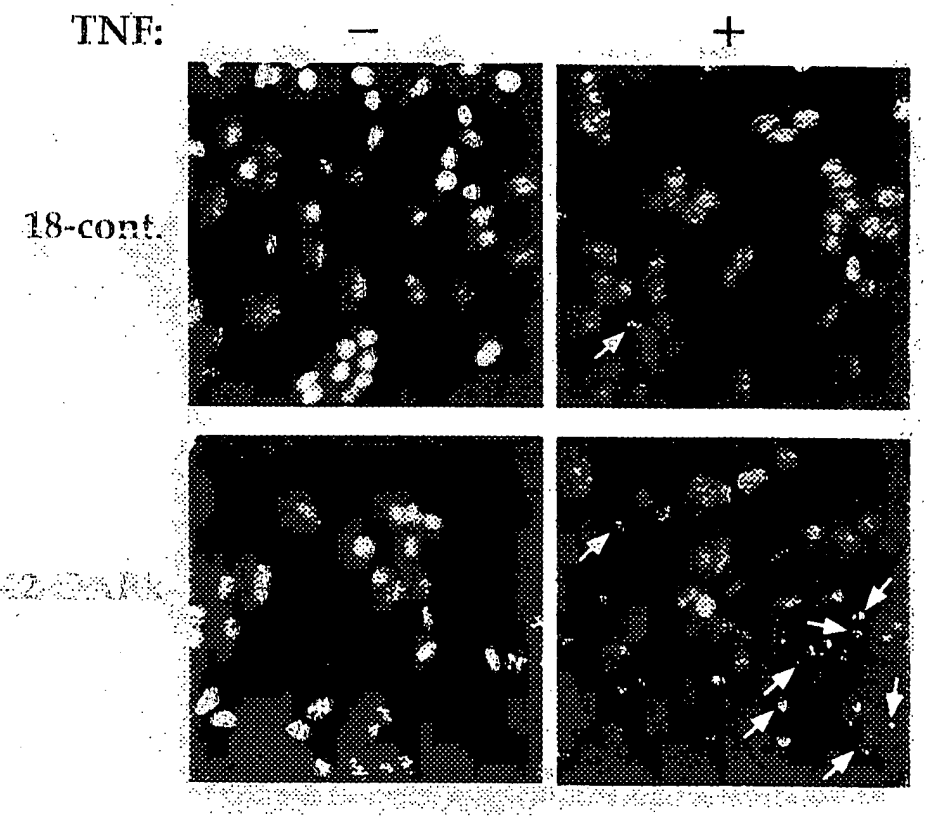


FIG. 26

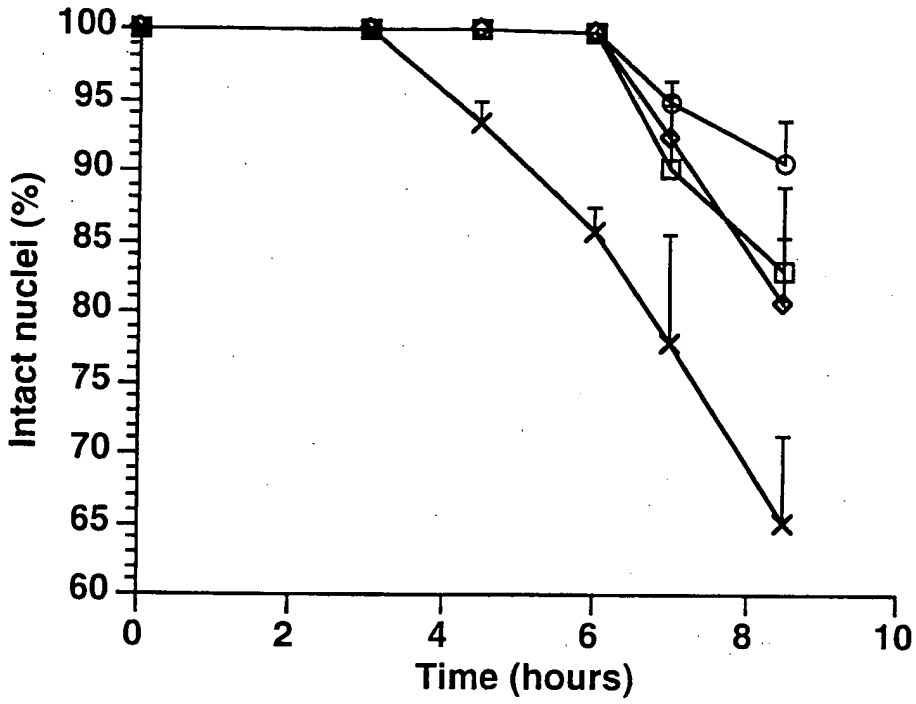


FIG. 27

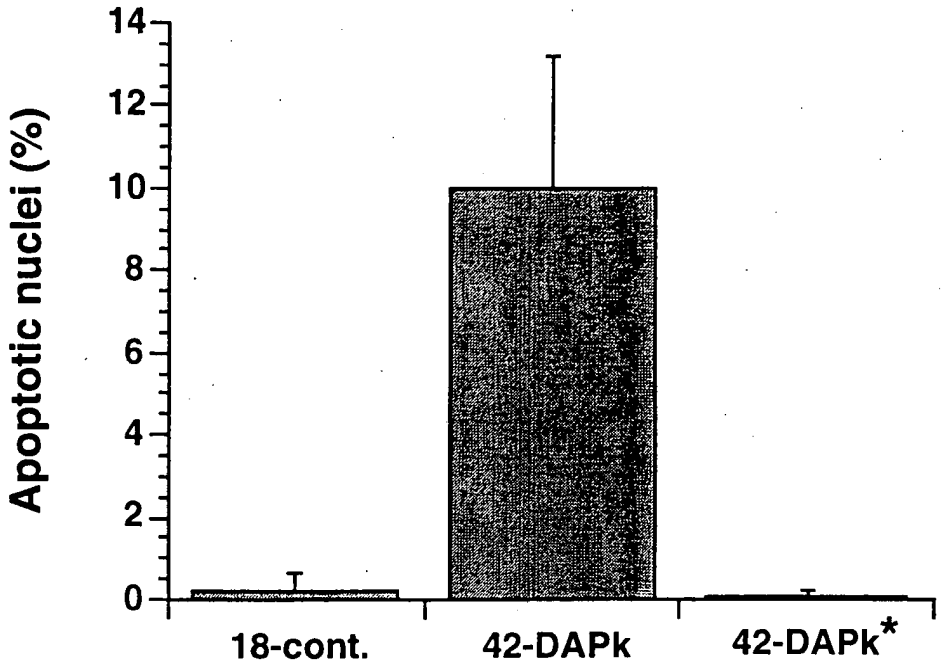


FIG. 28

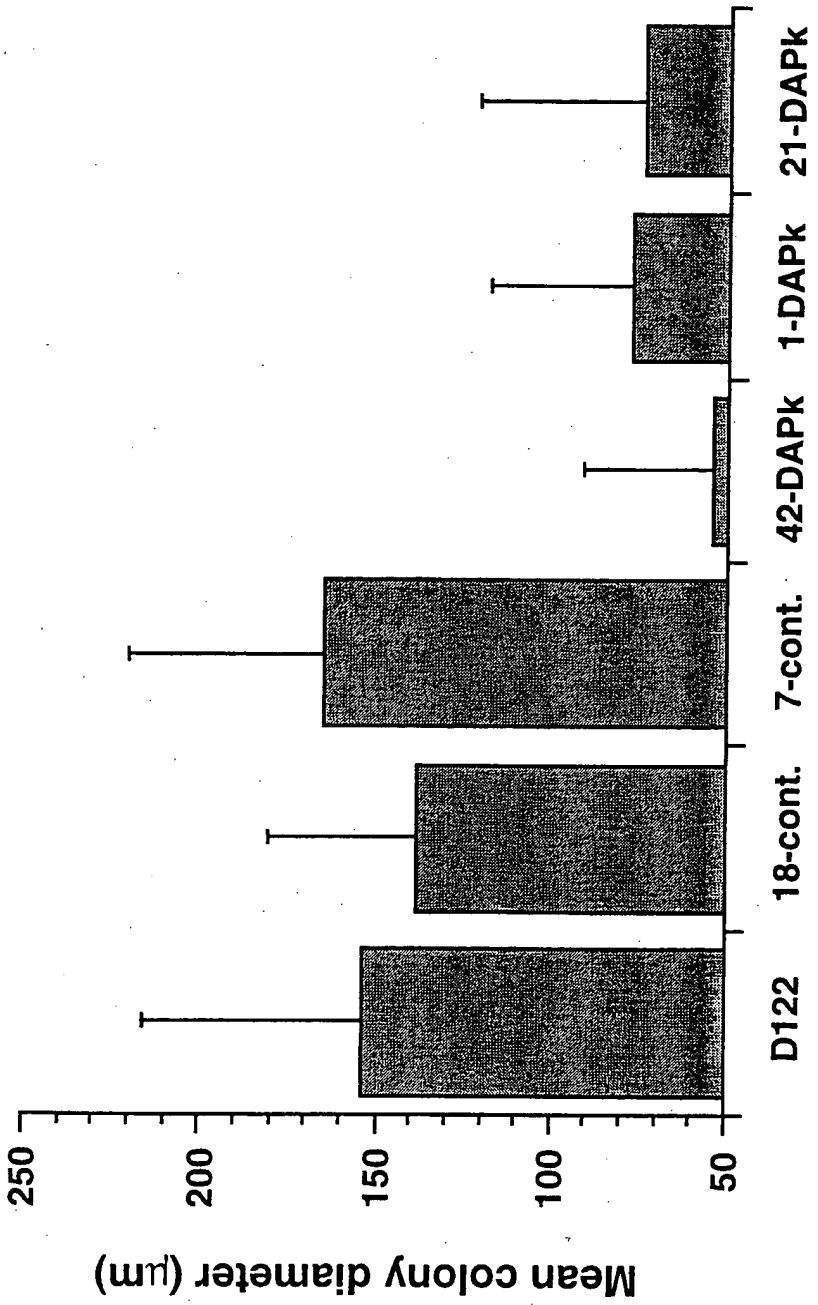


FIG. 29

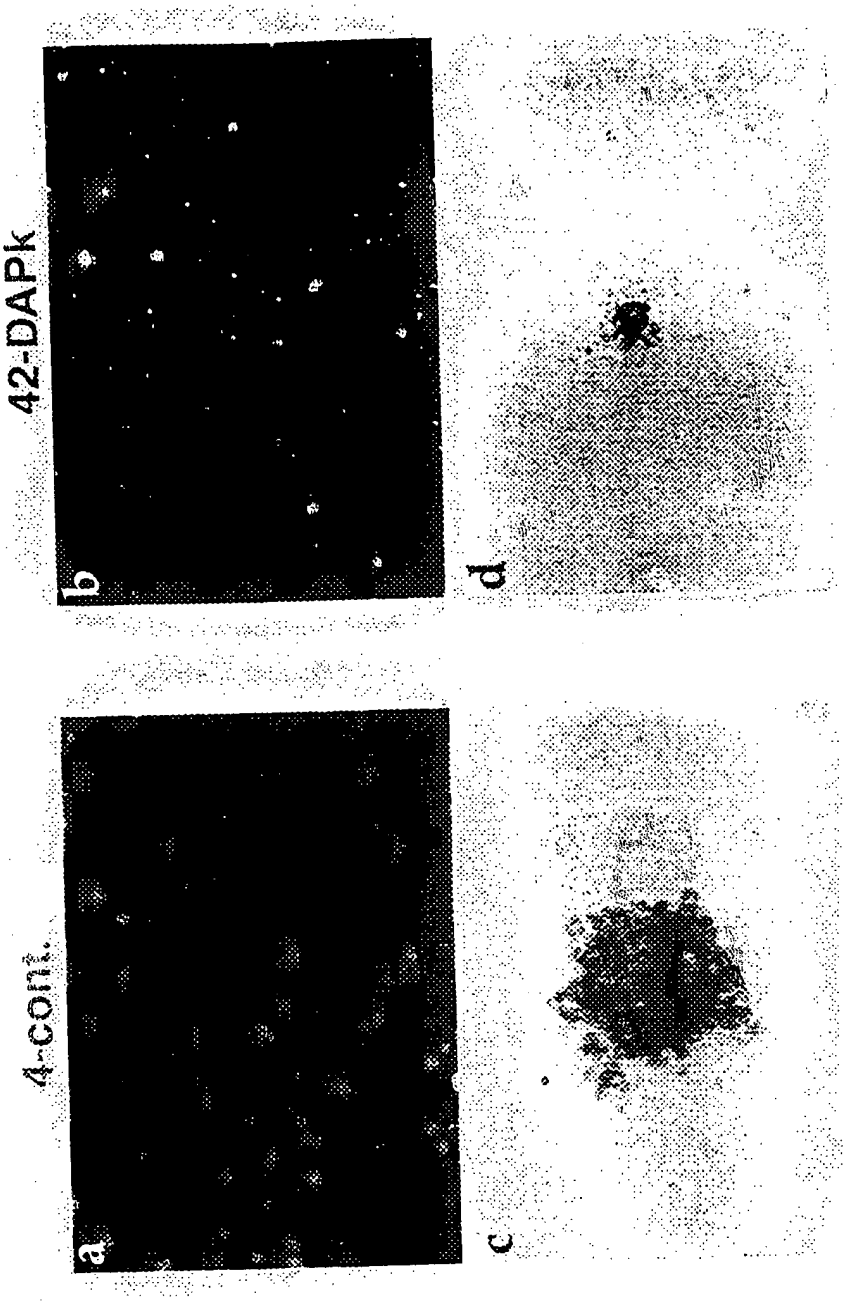


FIG. 30

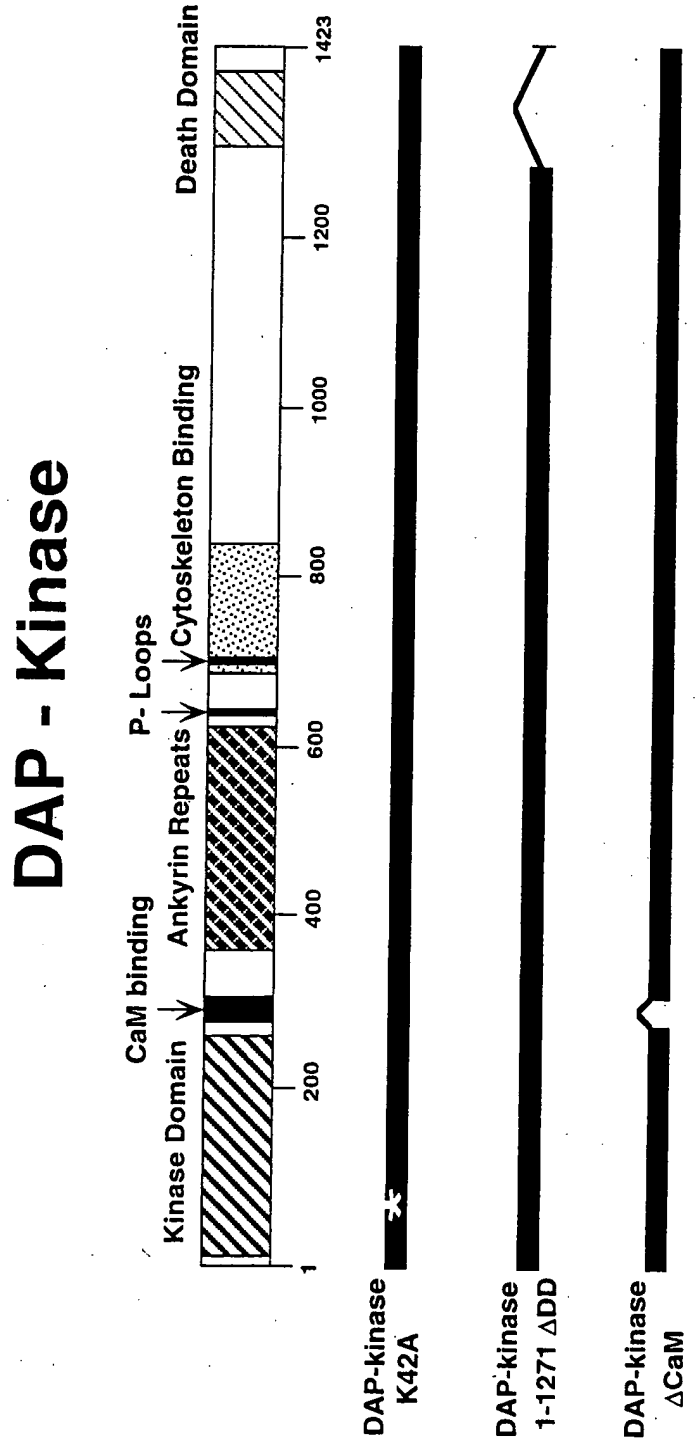


FIG. 31

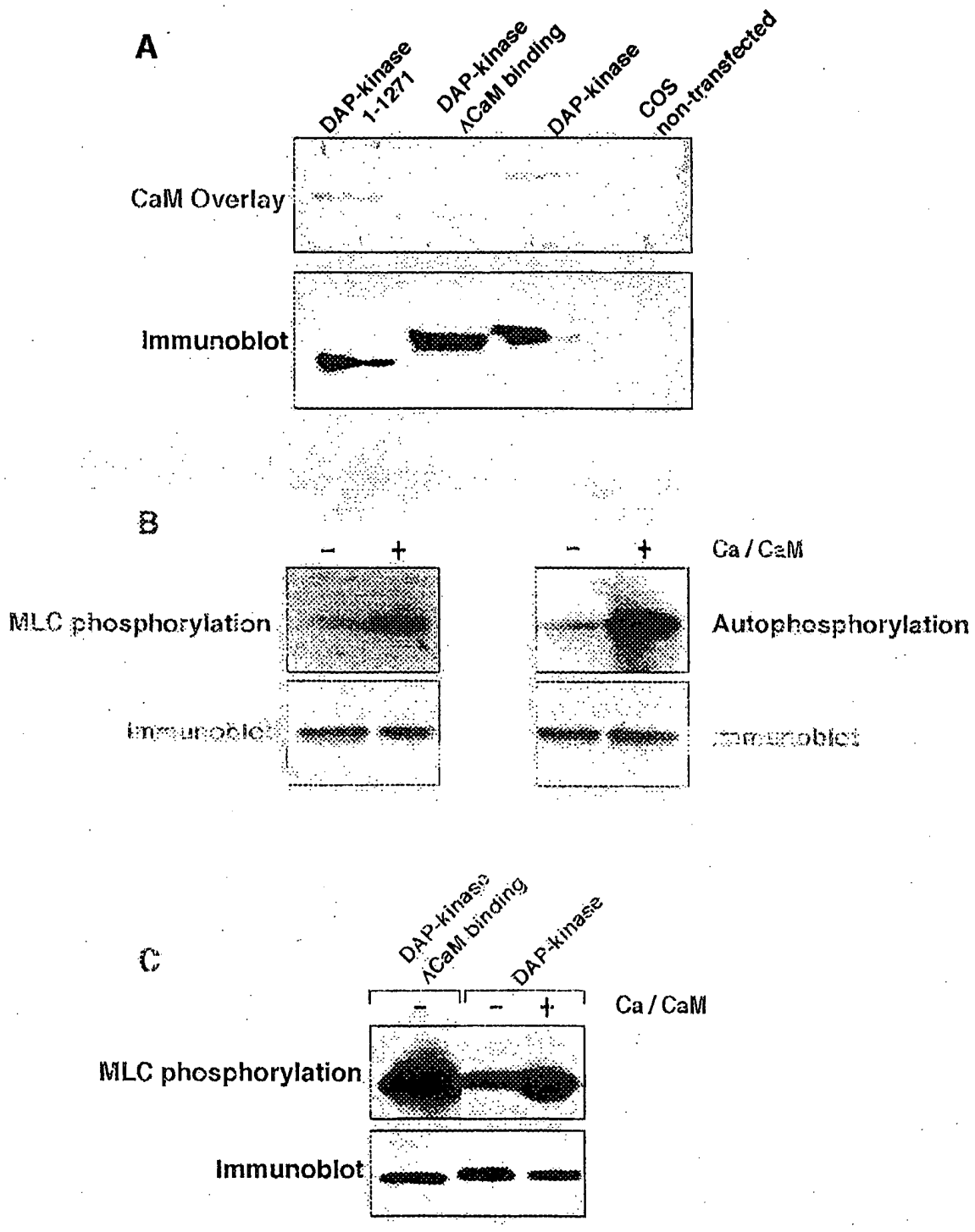


FIG. 33

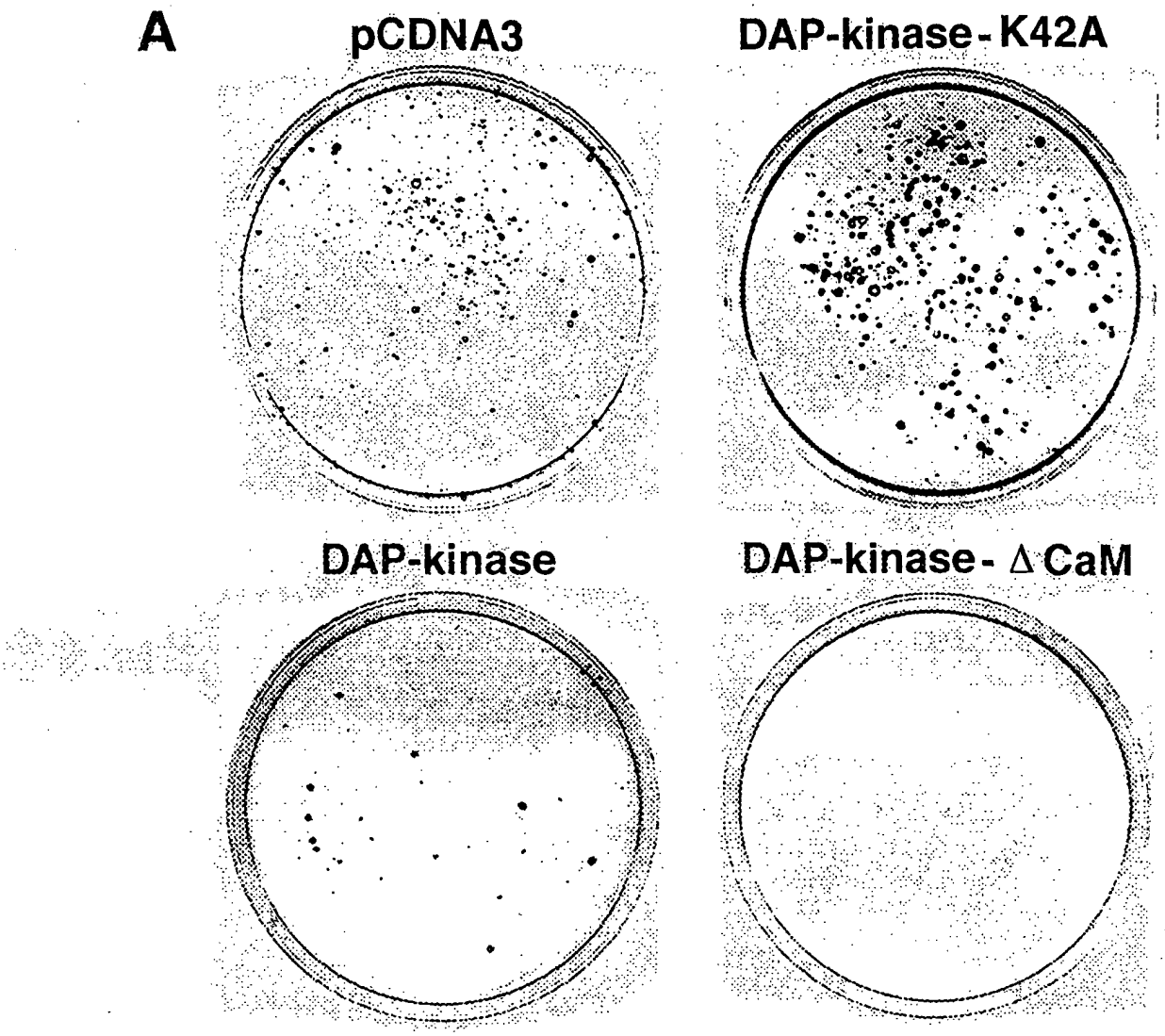


FIG. 34

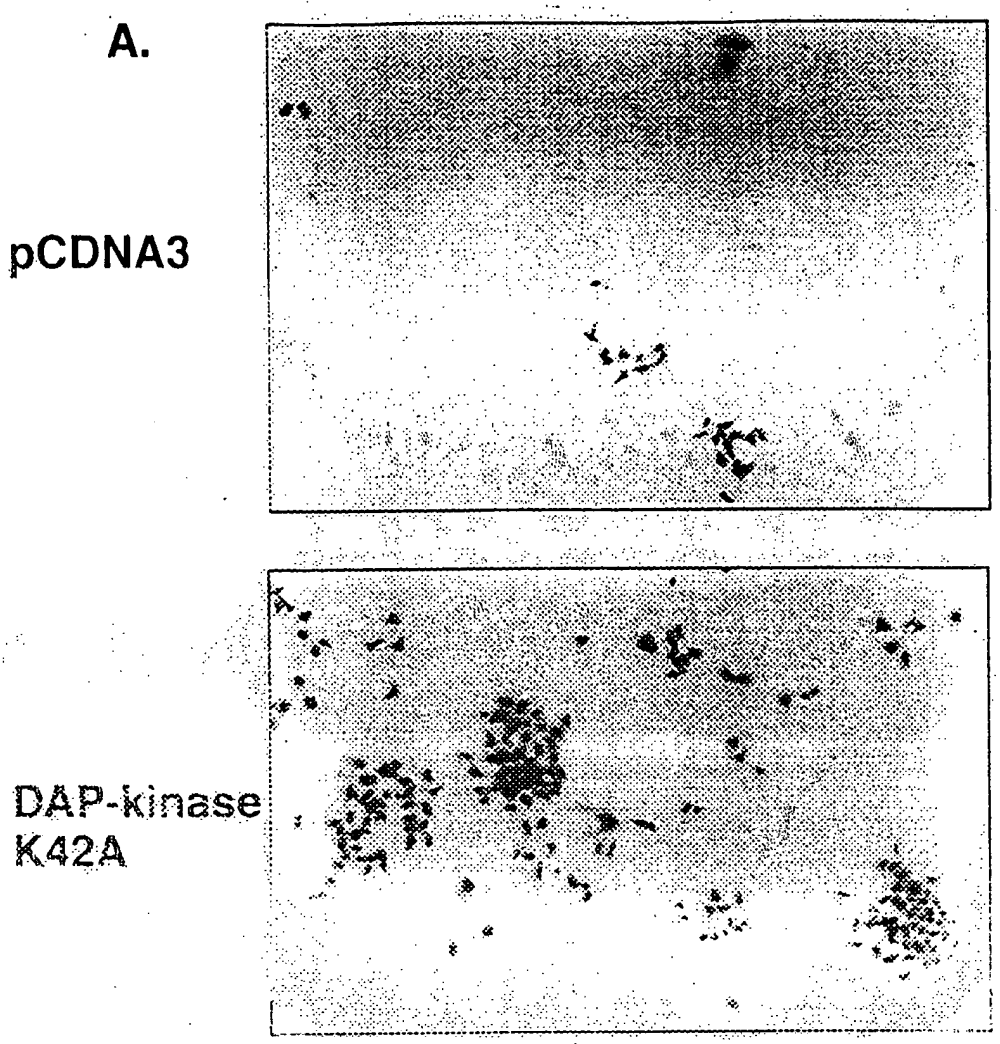


FIG. 35

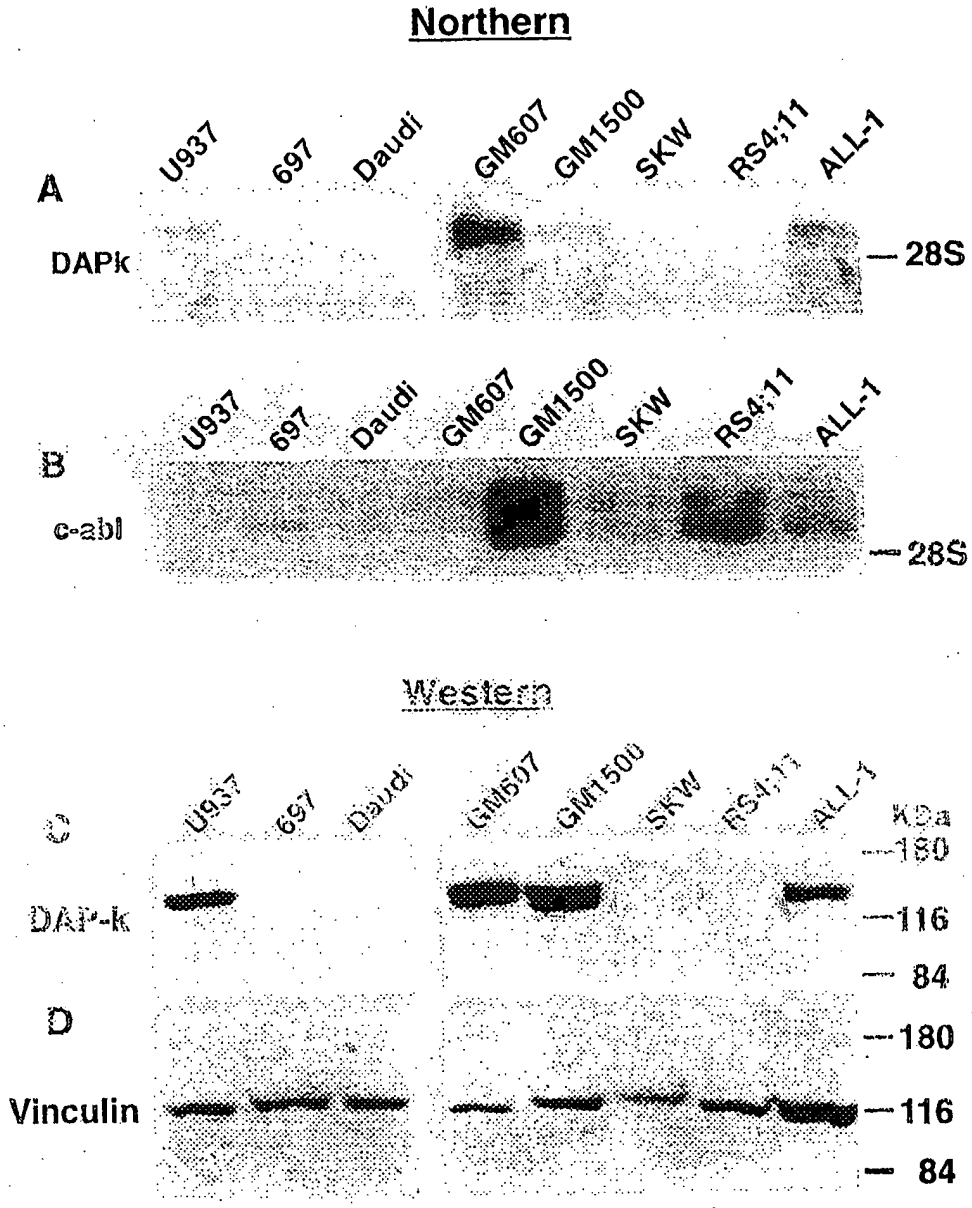


FIG. 36

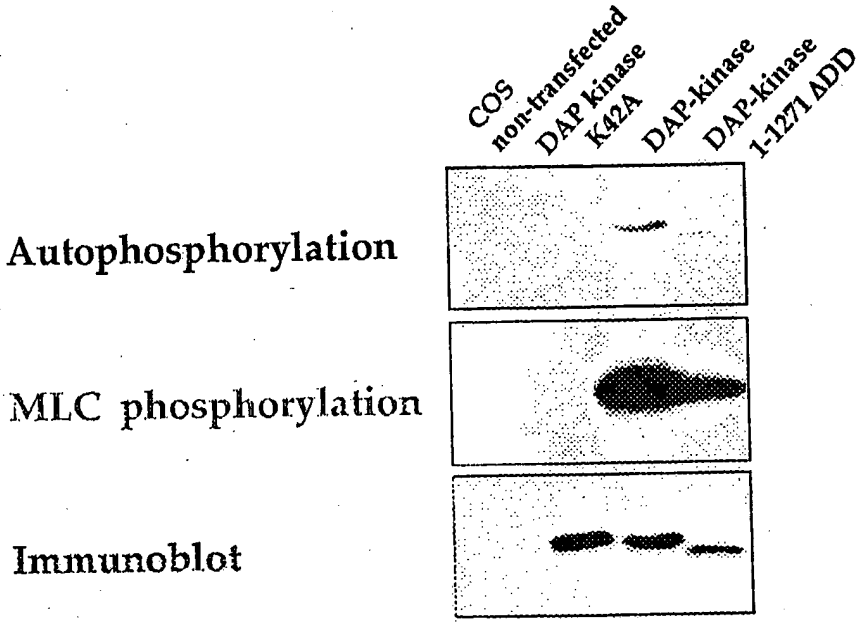


FIG. 32

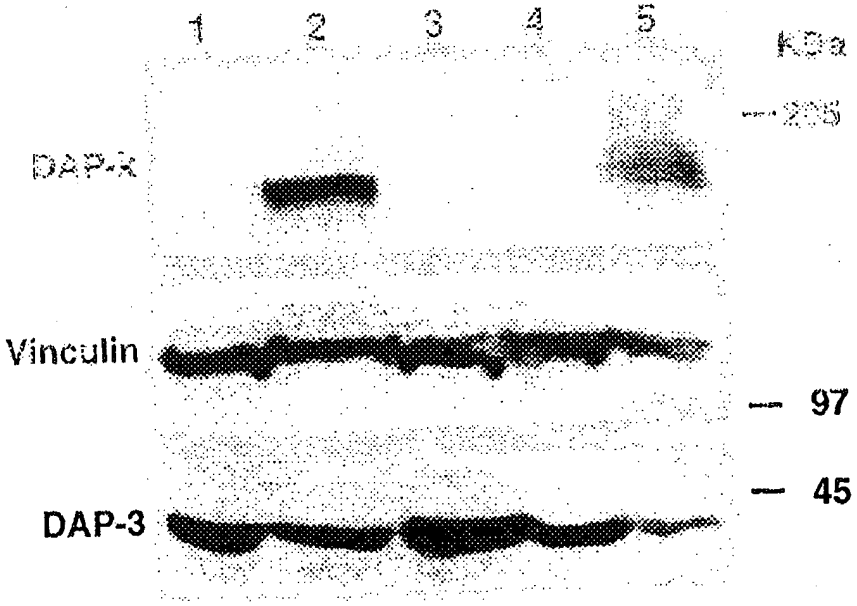


FIG. 37

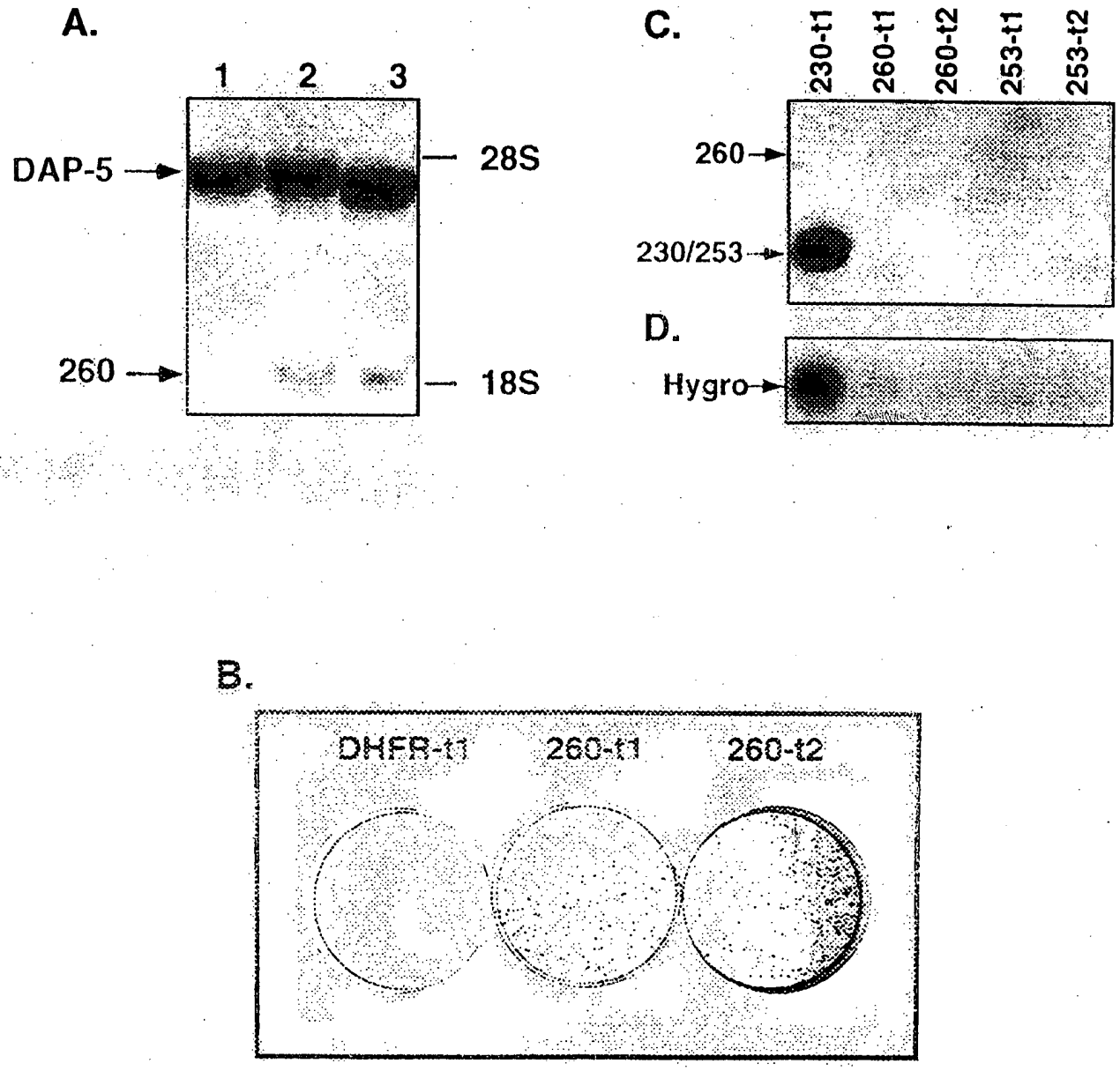


FIG. 38

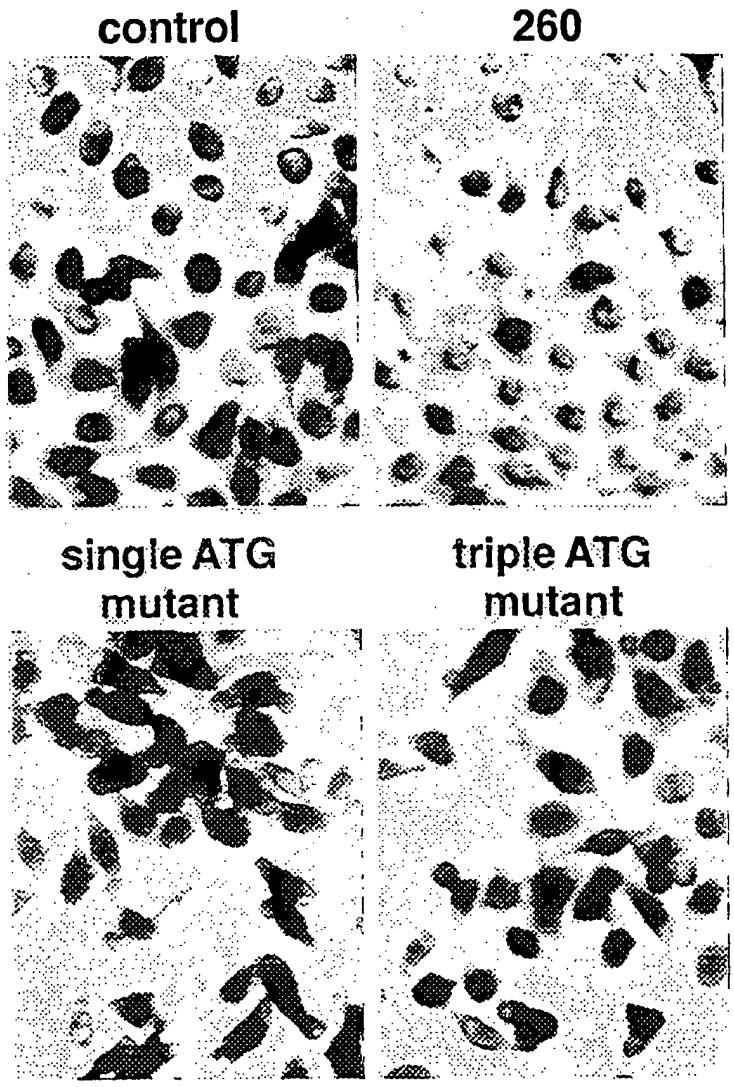
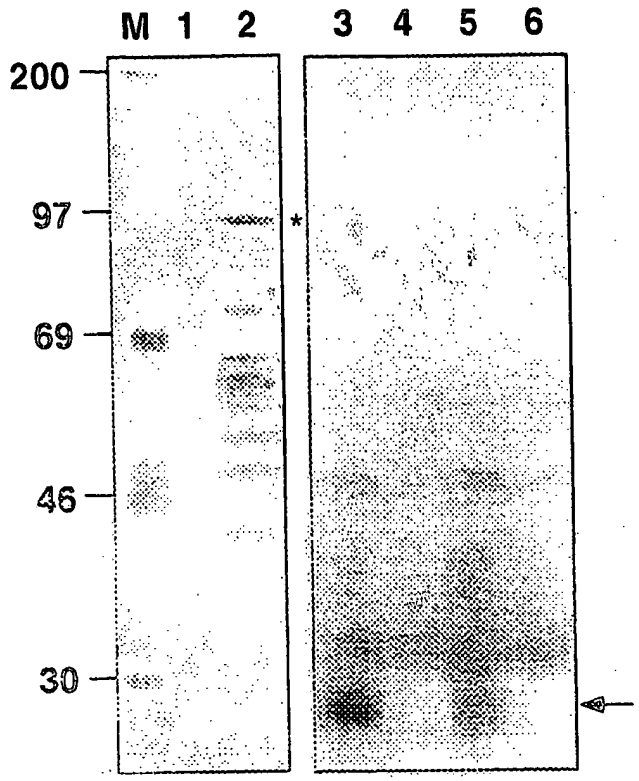


FIG. 39



B.

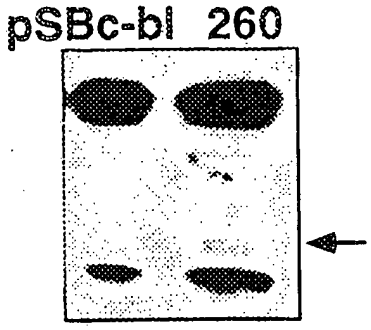


FIG. 40

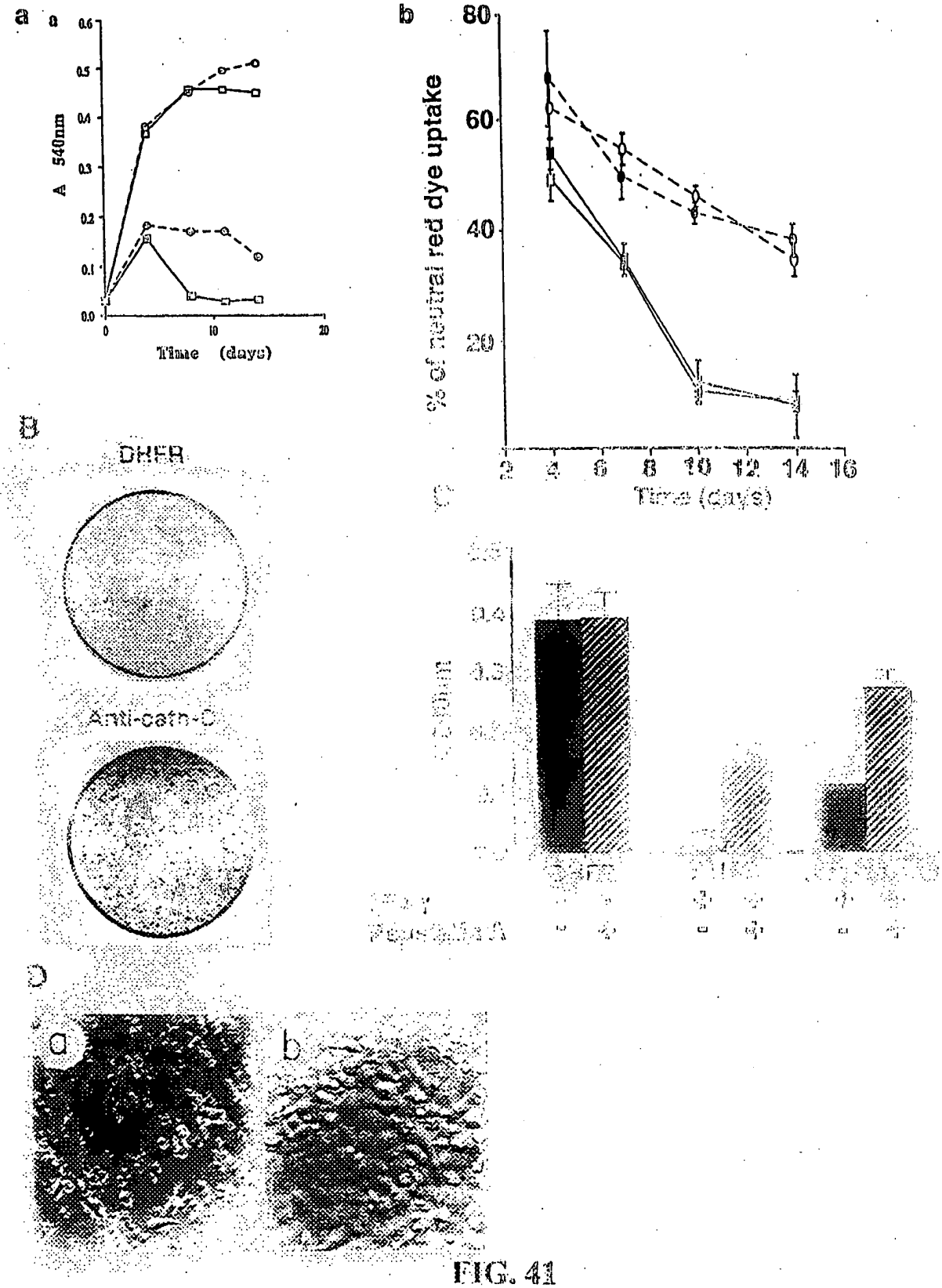


FIG. 41

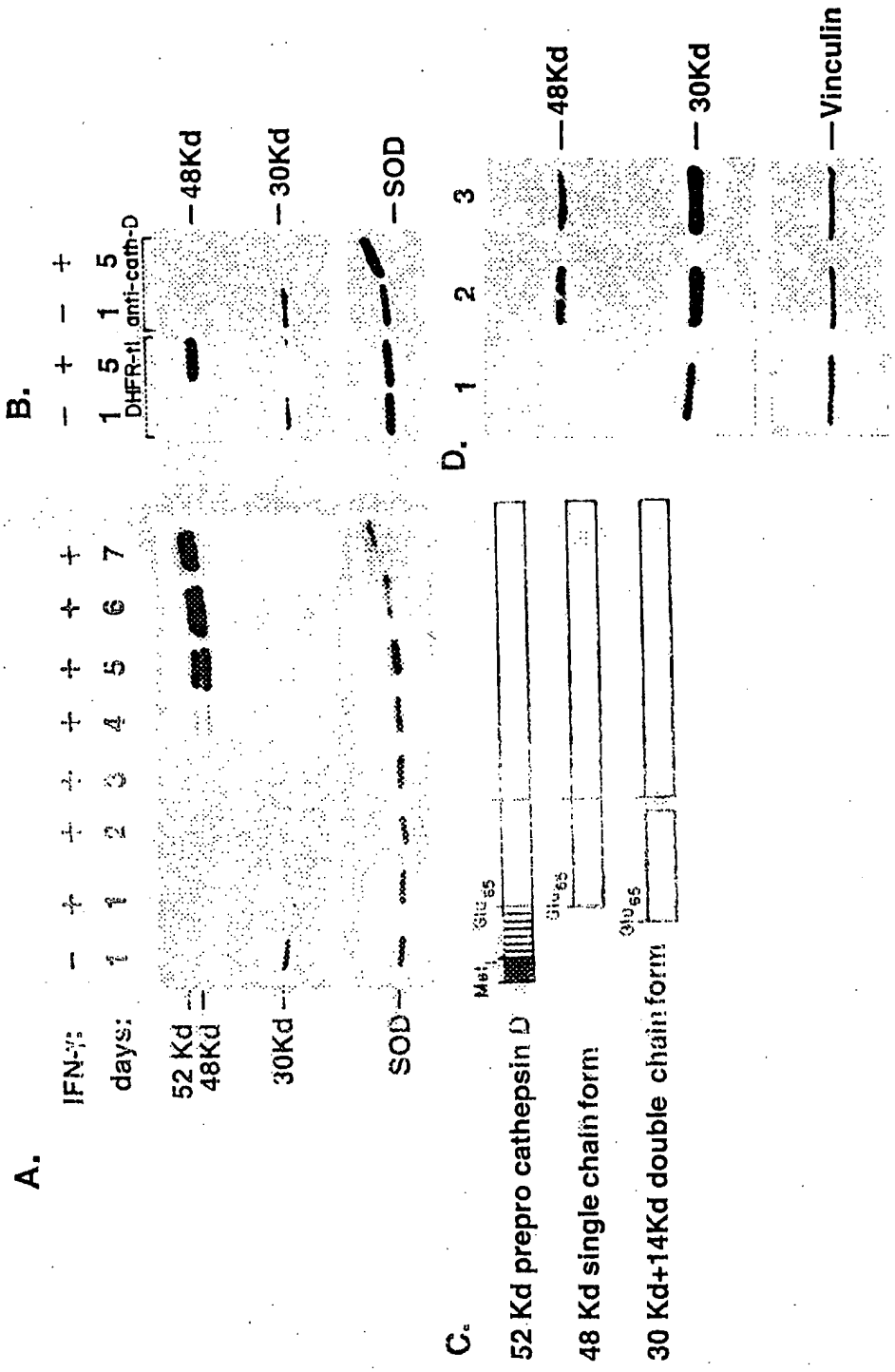


FIG. 42

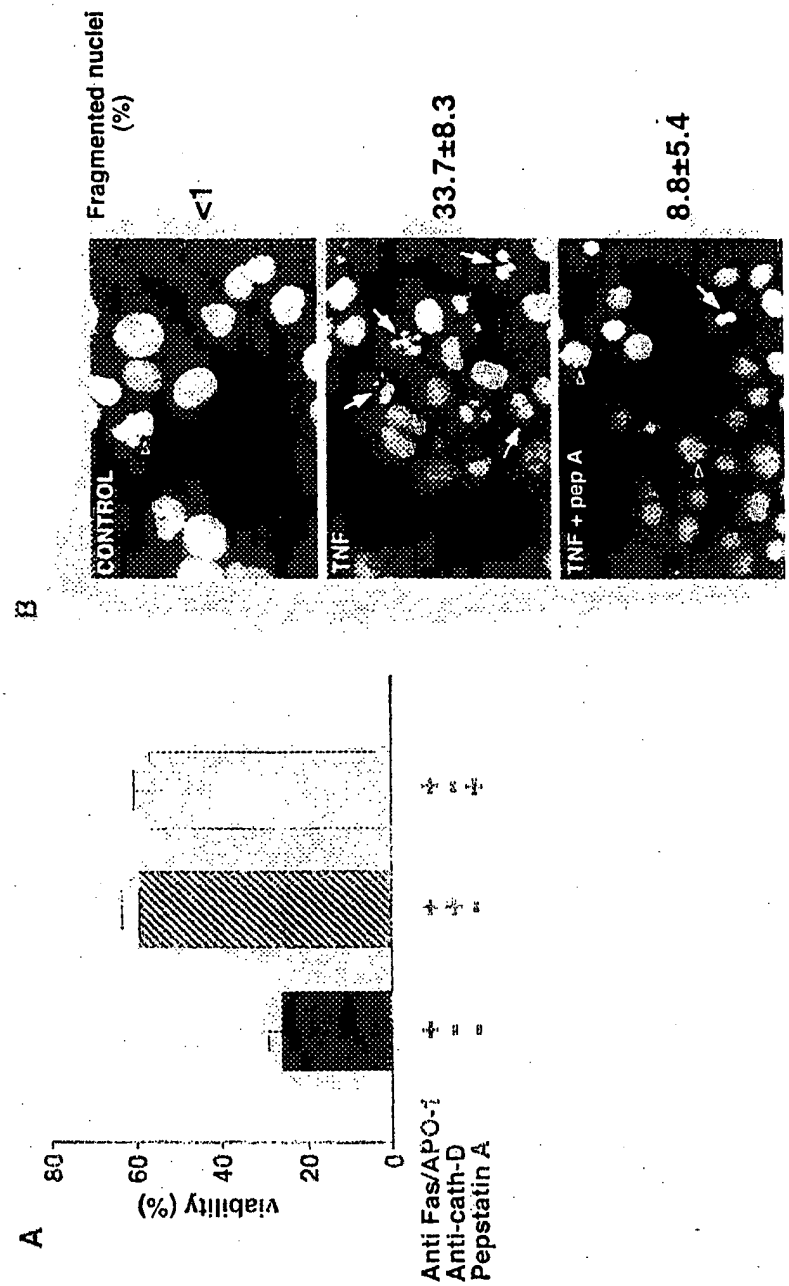


FIG. 43

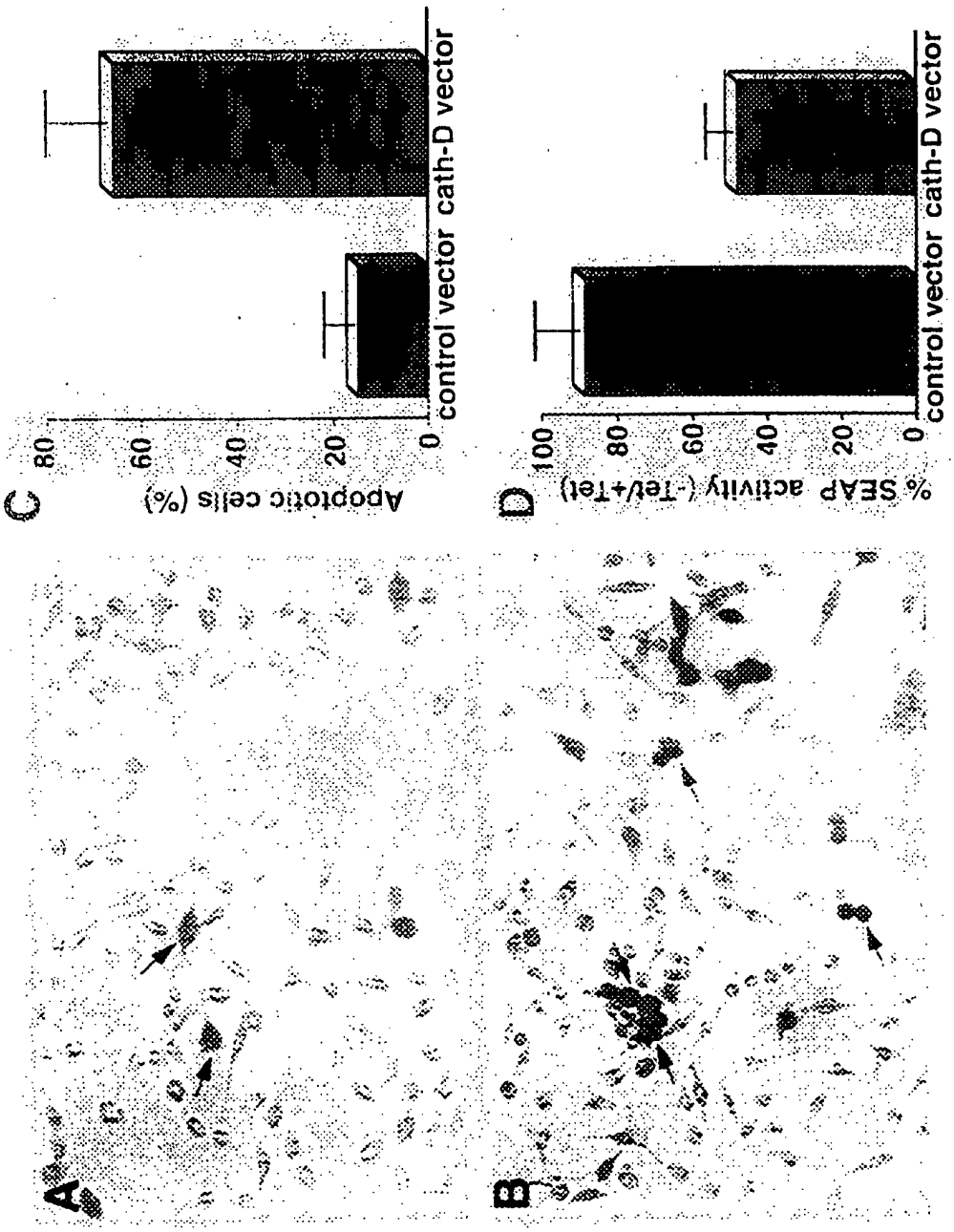


FIG. 44

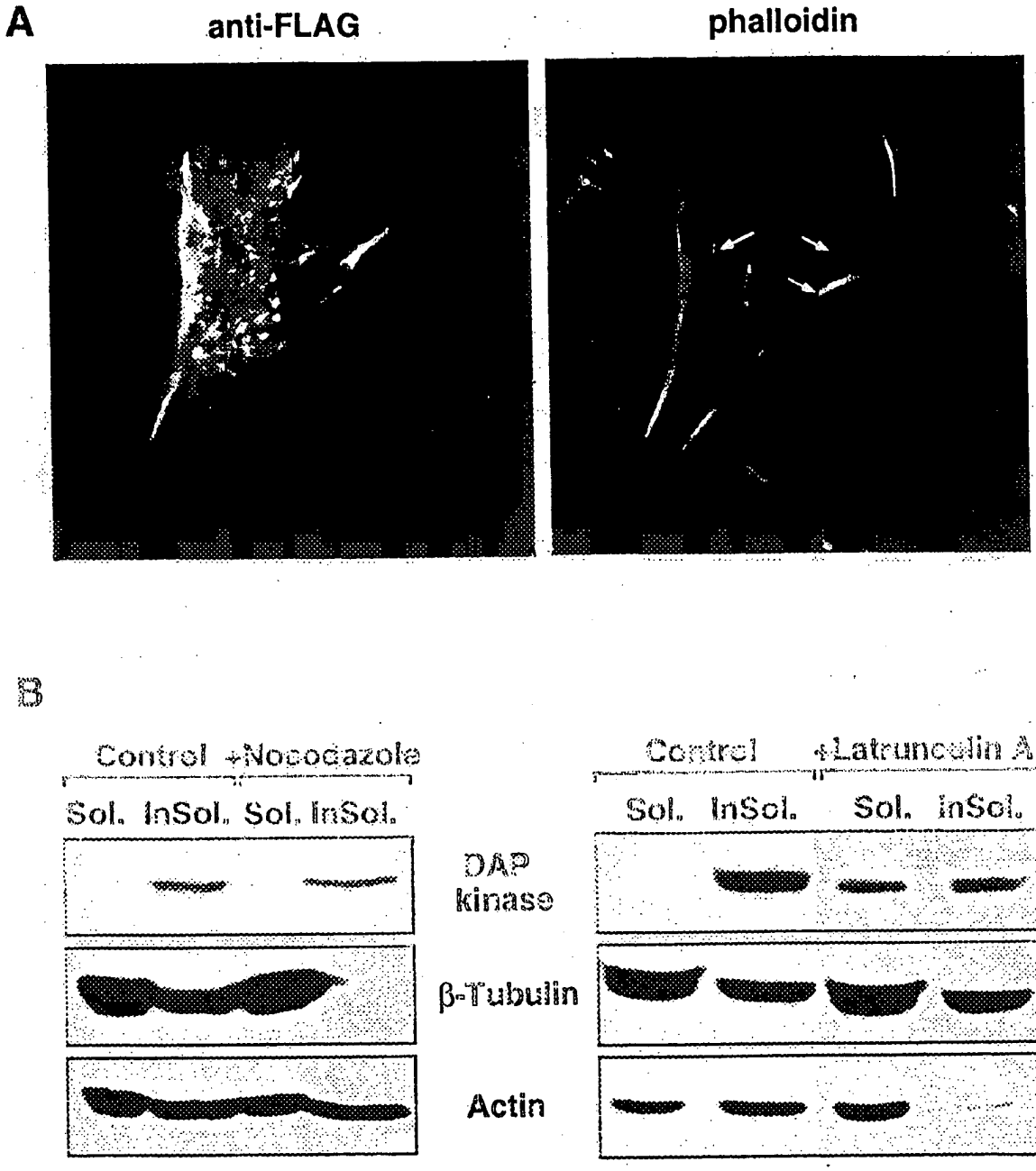


FIG. 45

A

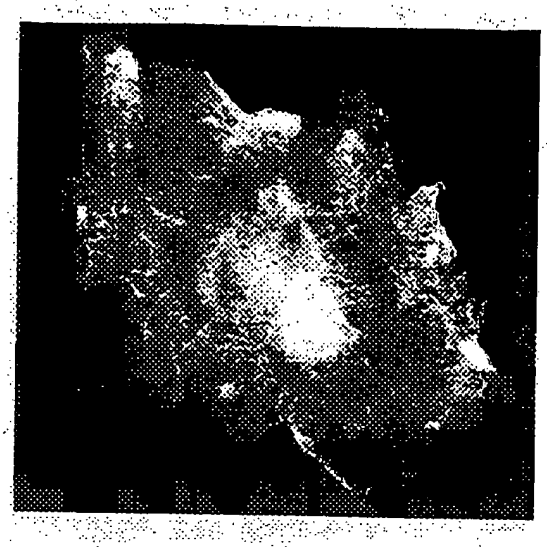


FIG. 46

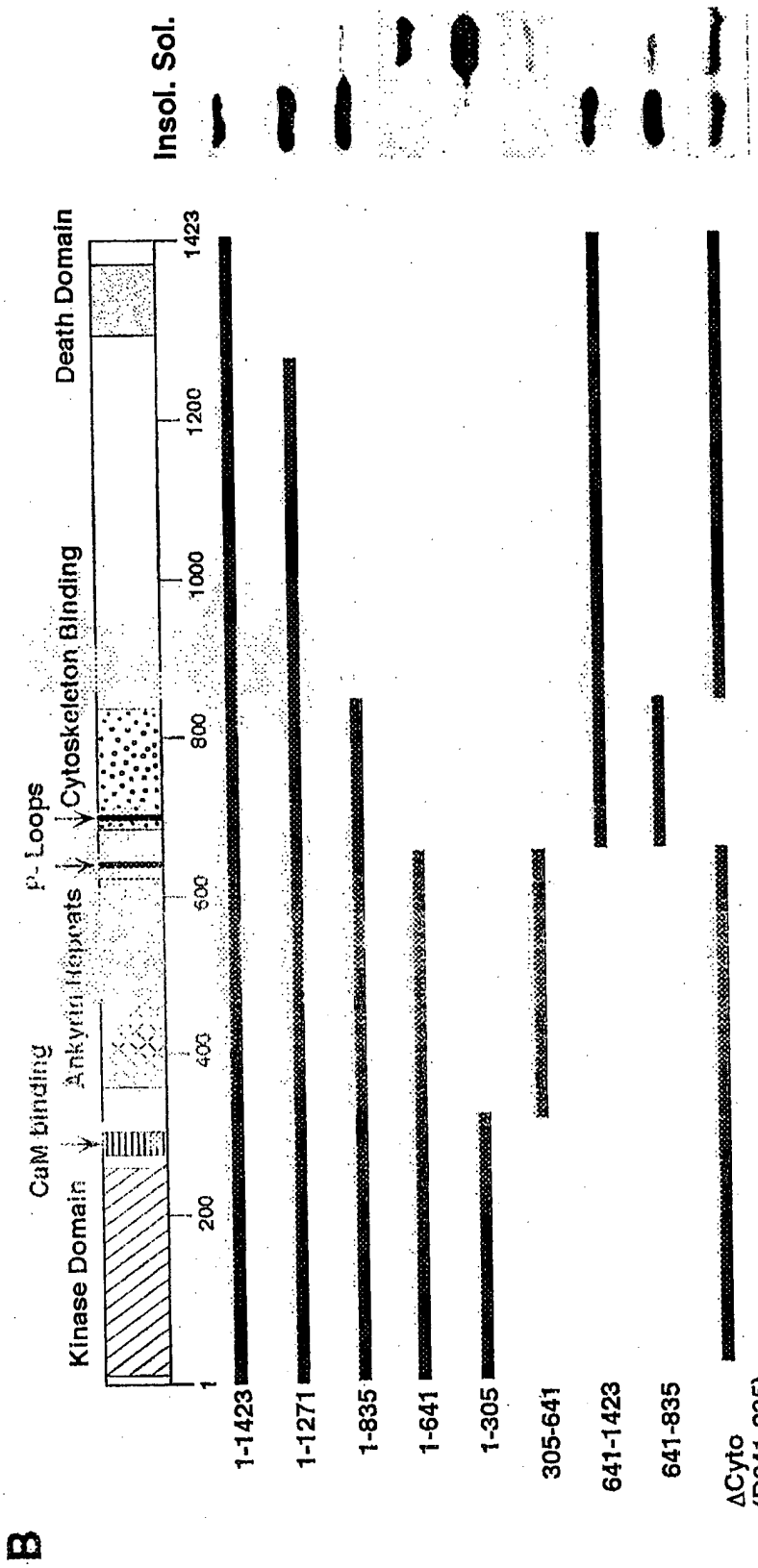


FIG. 46(cont.)

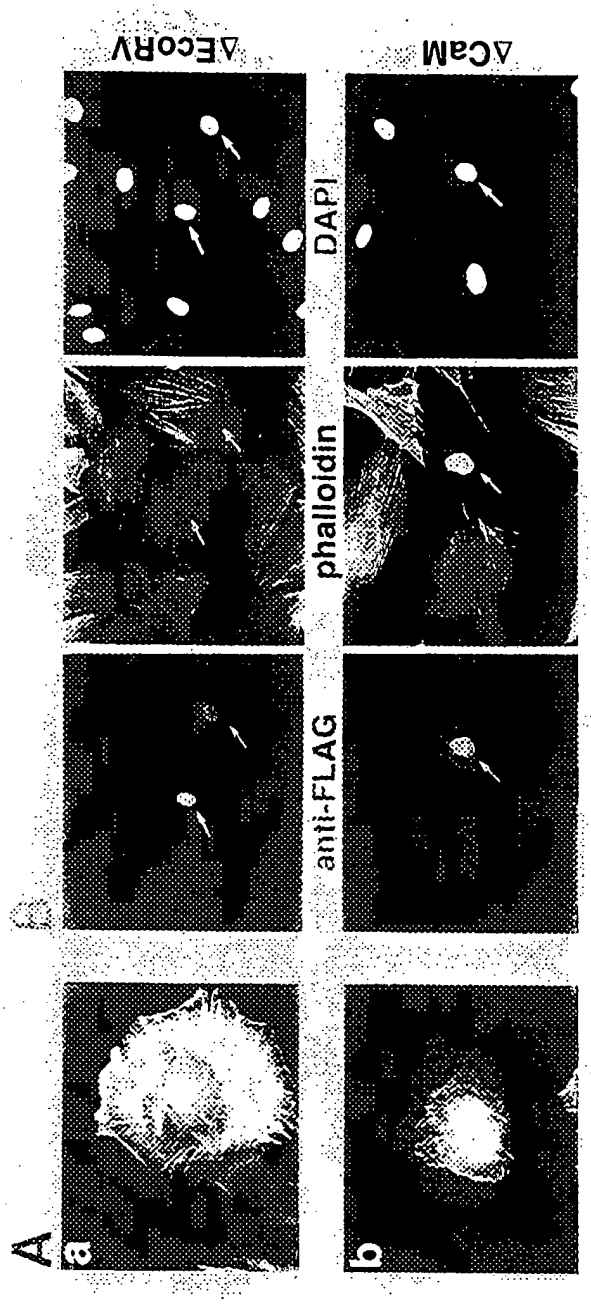


FIG. 47

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(51) International Patent Classification 6 :
C12N 15/11, 15/12, C07K 14/47, C12N 9/64, C12Q 1/68, A61K 48/00, C12N 9/12

A3

(11) International Publication Number: **WO 98/39429**

(43) International Publication Date: 11 September 1998 (11.09.98)

(21) International Application Number: PCT/IL98/00102

(22) International Filing Date: 3 March 1998 (03.03.98)

(30) Priority Data:
08/810,712 3 March 1997 (03.03.97) US

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(74) Agent: REINHOLD COHN AND PARTNERS; P.O. Box 4060, 61040 Tel Aviv (IL).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
10 December 1998 (10.12.98)

(54) Title: COMPOSITIONS FOR TREATMENT OF DISORDERS INVOLVING PROGRAMMED CELL DEATH

(57) Abstract

Use of a DNA sequence capable of inducing programmed cell death, in the preparation of a pharmaceutical composition for use in the treatment of a disease or a disorder associated with metastasizing pathological cell growth. Also described is the use of a DNA sequence capable of promoting non–cytokine–induced programmed cell death, in the preparation of a pharmaceutical composition useful in the treatment of a disease or a disorder associated with uncrontrolled pathological cell growth, or in the treatment of a disease or a disorder associated with non–cytokine induced programmed cell death.

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CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 98/00102

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C12N15/12 C07K14/47 C12N9/64 C12Q1/68
A61K48/00 C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12K C12N C12Q A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 10630 A (YEDA RESEARCH AND DEVELOPMENT CO. LTD.) 20 April 1995 pages 1-15; claims; figures * ---	1-10, 26-28
X	EP 0 781 844 A (OTSUKA PHARMACEUTICAL CO., LTD) 2 July 1997 * page 5, lines 4-12, 28, 56 * & WO 96 12017 A (OTSUKA PHARMACEUTICAL CO., LTD.) 25 April 1996 ---	8-10, 26-28
A	KISSIL, J.L. ET AL.: "Isolation of DAP3, ..." J. BIOL. CHEM., vol. 270, no. 46, 1995, pages 27932-27936, XP002080572 * whole disclosure * ---	1-10, 26-28

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 1998	23. 10. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hermann, R

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern...nal Application No
PCT/IL 98/00102

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FEINSTEIN, E. ET AL.: "Assignment of DAP1 and DAPK- ..." GENOMICS, vol. 29, 1995, pages 305-307, XP002080573 * p. 306, last paragraph *	8-10, 26-28

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 98/00102

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 11,21
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

1-10, 26-28 of the set of claims submitted under rule 26 with the date of 09 april 1998

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-7

   Use of an expression vector comprising a DNA sequence capable of inducing apoptosis for the preparation of a pharmaceutical composition for use in the treatment of disorders associated with metastasizing pathological cell growth.

2. Claims: 8-10,26-28

   Methods of determining the prognosis of a metastatic disease using nucleic acid probes against the DNA recited in claims 1-3 or 14-16, or using antibodies against the proteins encoded by said DNA.

3. Claim : 12

   A DNA comprising the sequence from position 1767 to position 2529 of fig. 15

4. Claim : 13

   Use of pepstatin A ... in the treatment of cathepsin D-related programmed cell death

5. Claims: 14-20, 29,30

   Use of an expression vector comprising a DNA sequence capable of promoting non-cytokine-induced programmed cell death for the preparation of a pharmaceutical composition for use in the treatment of disorders associated with uncontrolled pathological cell growth.

6. Claims: 22-25, 29,30

   Use of an expression vector comprising a DNA capable of inhibiting non-cytokine induced programmed cell death ... in the treatment of a disorder associated with non-cytokine induced programmed cell death.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 11,21

Claims 11 and 21: Said claims are drafted to the result to be achieved ("... a mutation resulting in ... increased ... activity"); they do not contain any information concerning the technical nature of said mutation. Technical features are however necessary for a meaningful search.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr....onal Application No
PCT/IL 98/00102

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9510630 A	20-04-1995	CA 2174136 A	20-04-1995
		EP 0804613 A	05-11-1997
		JP 9505992 T	17-06-1997
EP 781844 A	02-07-1997	AU 3673395 A	06-05-1996
		CA 2202628 A	25-04-1996
		CN 1171133 A	21-01-1998
		WO 9612017 A	25-04-1996